Functional and mechanistic studies on catalase-peroxidase

Doctoral Thesis

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meinen Eltern
allen ehemaligen und zukünftigen
DissertantenInnen
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Appendix one

Intracellular catalase/peroxidase from the phytopathogenic fungus Magnaporthe grisea: expression analysis and biochemical characterization of the recombinant protein

Appendix two

Protein- and solvent-based contributions to the redox thermodynamics of lactoperoxidase and eosinophil peroxidase
Abstract

Catalase-peroxidases (KatGs) are unique bifunctional enzymes with predominant catalatic and substantial peroxidatic activity found in Bacteria, Archaea and Eukarya. Together with ascorbate peroxidases (APX) and cytochrome c peroxidases (CcP) they belong to class I of the superfamily of plant, bacterial, and fungal heme-containing peroxidases (EC 1.11.1.7). Despite their striking sequence and structural similarities to APX and CcP KatGs are the only heme peroxidases capable of both reduction and efficient oxidation of hydrogen peroxide with rates similar to typical (monofunctional) catalases. The present work clearly demonstrated that H₂O₂ dismutation to water and molecular oxygen in KatG follows a non-scrambling mechanism with the two oxygen atoms in the evolving O₂ coming from the same H₂O₂ molecule. Gas chromatography–mass spectrometry analysis using ¹⁶O- and ¹⁸O-labeled H₂O₂ showed the formation of ¹⁸O₂ (m/e = 36) and ¹⁶O₂ (m/e = 32) but not ¹⁶O¹⁸O (m/e = 34) indicating that in the two-electron oxidation step of H-O-O-H the O–O bond is not cleaved. Apart from catalyzing the same overall dismutation reaction (2 H₂O₂ → 2 O₂ + 2 H₂O) as monofunctional catalases, catalase-peroxidases follow an alternative catalatic mechanism. Distinct differences between bovine liver catalase and KatG regarding spectral features of redox intermediates could be monitored using multi-mixing stopped-flow UV-Vis spectroscopy. Depending on pH two different low-spin intermediates that dominate during H₂O₂ degradation could be trapped. Recent rapid-freeze-quench electronic spin resonance spectroscopy demonstrated the formation of a protein-based radical on the KatG-typical distal adduct that persists during peroxide turnover. This peculiar post-translational modification in the active site of KatG - consisting of covalently linked Trp122, Tyr249 and Met275 (Synechocystis numbering) - rapidly reduces the porphyril cation radical usually found in redox intermediates (compound I) of heme peroxidases. In the presence of millimolar hydrogen peroxide KatG is oxidized to compound I that rapidly converts to oxoferrous heme (compound III) that co-exists with the radical formed at the covalent adduct. The latter guarantees both the release of O₂ as well as rapid turnover of compound III to ferric KatG. In (monofunctional) peroxidases, however, the decay of compound III is extremely slow and superoxide is released instead of O₂. The importance of the Trp122-Tyr249-Met275 adduct in
the bifunctional activity was reflected by the fact that mutation of either Trp122 or Tyr249 completely converted a bifunctional KatG to a monofunctional peroxidase. In order to follow the H₂O₂ oxidation reaction by both KatG and monofunctional catalases a new method was developed that avoids enzyme cycling after substrate addition, which is a general phenomenon in dismutating oxidoreductases. In the sequential stopped-flow study the heme proteins were oxidized to compound I by peroxoacetic acid and finally mixed with H₂O₂ in the presence of cyanide. The latter inhibited enzyme cycling by trapping the proteins in their low-spin complexes with Kᵋ values in the micromolar region. This, for the first time enabled calculation of apparent bimolecular H₂O₂ oxidation rates of both KatG and a monofunctional catalase. In case of KatG the rates most probably reflect the transition of oxyferrous KatG to the ferric form. This study underlined the proposed catalatic mechanism and clearly showed that the transition of compound III to native KatG is more than 3 orders of magnitude faster than in monofunctional peroxidases.

Another structural feature, apart from the covalent adduct, that enables KatG to be catalatically active, is the long and narrow substrate channel with an extensive and rigid H-bonding network. Interestingly, similar to manipulation of the distal adduct, mutations of residues involved in this H-bonding network decrease the catalase activity whereas the peroxidase activity remains unaffected or is even enhanced. Important residues for maintenance of this ordered matrix of water molecules were shown to be the catalytic residues His123 and Arg119, as well as Asp152 (controls channel entrance to heme cavity) and Glu253 (entrance of main substrate channel). This was demonstrated by kinetic analysis in combination with temperature dependent spectroelectrochemical investigations. The latter study gave a valuable inside in the redox thermodynamics of KatG and underlined the importance of the channel architecture for KatG catalysis.

Finally, based on structural analysis and multiple sequence alignment, a putative peroxidase substrate binding and oxidation site was analysed by site-directed mutagenesis and kinetic investigations. Various mutants were designed and probed for oxidation of the artificial substrates guaiacol or ABTS that are known to be oxidized by KatG but are too large for entering the main access channel. Unfortunately, manipulation of this putative binding site did not significantly alter the bifunctional activity of KatG. Thus, both the nature of the endogenous peroxidase substrate as well as its binding site remain unknown.
Abstract

Katalase-Peroxidasen (KatGs) sind bifunktionale Oxidoreduktasen (EC 1.11.1.7) mit vorwiegend katalatischer, aber auch beachtlicher Peroxidaseaktivität. Sie gehören gemeinsam mit Ascorbat-Peroxidasen (APX) und Cytochrom c Peroxidasen (CcP) zur Klasse I der Superfamilie Häm-haltiger Peroxidasen aus Pflanzen, Pilzen und Bakterien. Trotz ihrer Homologie und strukturellen Ähnlichkeit mit APX und CcP, sind KatGs die einzigen Peroxidasen mit nennenswerter Katalaseaktivität und daher ideale Modellenzyme für das Studium der enzymkatalysierten Wasserstoffperoxid-Dismutation.

In dieser Arbeit konnte mittels Gaschromatographie in Kombination mit Massenspektrometrie gezeigt werden, dass im Zuge der Dismutation von $^{16}$O- und $^{18}$O-markierter H$_2$O$_2$ $^{18}$O$_2$ ($m/e = 36$) und $^{16}$O$_2$ ($m/e = 32$), aber kein $^{16}$O$^{18}$O ($m/e = 34$) gebildet wird. Dies zeigt, dass bei der Oxidation des zweiten Peroxidmoleküls die O-O Bindung intakt bleibt. Weiters konnte durch Stopped-flow Spektroskopie in Kombination mit Elektronenspinresonanz-Spektroskopie die elektronische Natur der im Katalasezyklus involvierten Redoxintermediate geklärt und somit erstmals ein plausibler Reaktionsmechanismus für die Gesamtreaktion (2 H$_2$O$_2$ $\rightarrow$ 2 O$_2$ + 2 H$_2$O) postuliert werden, der dem KatG-spezifischen distalen kovalenten Addukt Trp122-Tyr249-Met275 die zentrale Rolle zuweist. Ähnlich wie in monofunktionalen Peroxidasen durchläuft auch KatG in Gegenwart millimolarer Konzentration die Redoxintermediate Compound I und Compound III. Im Zuge dieser Redoxkaskade wird jedoch in KatG rasch ein Oxidationsäquivalent vom Porphyrinring zum Addukt verschoben und dies garantiert eine effiziente und rasche Freisetzung von O$_2$.

Die Kinetik der H$_2$O$_2$ Oxidationsreaktion konnte erstmals durch eine neue Methode analysiert werden. Katalase-Peroxidase wurde mittels Peressigsäure zu Compound I oxidiert und dieses Redoxintermediat im sequenziellen Stopped-flow Modus mit H$_2$O$_2$ in Gegenwart von Cyanid inkubiert. Dadurch kommt es zur Ausbildung eines stabilen low-spin Komplexes der gebildeten Fe(III) Form von KatG und die Folgereaktion im Katalasezyklus wird unterdrückt. Dadurch konnten erstmals sowohl für KatG als auch für eine monofunktionale Katalase die H$_2$O$_2$-Oxidationsraten bestimmt werden. Zudem wurde die Bedeutung des Adduktradikals dadurch unterstrichen, dass in monofunktionalen Peroxidasen zwar dieselbe Reaktionsfolge beschränkt wird, jedoch durch das Fehlen der KatG-spezifischen post-translationalen Modifikation der Übergang Compound III zum
nativen Enzym um mehr als drei Zehnerpotenzen langsamer abläuft, abgesehen davon dass Superoxid und nicht molekularer Sauerstoff freigesetzt wird. Weiters konnte gezeigt werden, dass – neben dem distalen kovalenten Addukt – die Architektur des Substratkanals für die effiziente $\text{H}_2\text{O}_2$ Oxidation entscheidend ist. Im Gegensatz zu monofunktionalen Peroxidasen ist der Substratkanal in KatG eng und lang und zeichnet sich durch ein ausgedehntes und rigides H-Brückennetzwerk aus. Mittels ortsspezifischer Mutagenese, kinetischer Analyse und Temperatur-abhängiger spektroelektrochemischer Untersuchungen, konnte klar gezeigt werden, dass nur die Oxidation aber nicht die Reduktion von $\text{H}_2\text{O}_2$ bei Manipulation dieses Substratkanals in Mitleidenschaft gezogen wird. Wichtig für die Stabilisierung dieser geordneten Wassermatrix sind die katalytischen Aminosäuren His123 und Arg119, als auch Asp152 (reguliert den Eintritt des Kanals in das aktive Zentrum) und Glu253 (Substratkanaleingang). Neben Trp122-Tyr249-Met275 (Addukt) sind diese Aminosäuren in prokaryotischen und eukaryotisch KatGs hoch konserviert.

introduction

Bifunctional catalase-peroxidases
Bifunctional catalase-peroxidases

Introduction

Hydrogen peroxide is one of the most frequently occurring reactive oxygen species in the biosphere. It emerges either in the environment or as a by-product of aerobic metabolism, i.e. by oxygen activation (e.g. superoxide formation and dismutation) in respiratory and photosynthetic electron transport chains and as product of enzymatic activity, mainly by oxidases. Both, excessive hydrogen peroxide as well as its decomposition product hydroxyl radical that is formed in a Fenton-type reaction, are harmful for almost all cell components. Thus, its rapid and efficient removal is of essential importance for all aerobically living prokaryotic and eukaryotic cells. On the other hand, hydrogen peroxide can act as a second messenger in signal transduction pathways, mainly for immune cell activation, inflammation, cellular proliferation and apoptosis. There is evidence that this signaling function of hydrogen peroxide was acquired rather late in evolution. In unicellular organisms H$_2$O$_2$ mainly stimulates production of antioxidants and ROS-removing and repairing enzymes, whereas in multicellular organisms (both animals and plants) it is involved in activation of signaling pathways.

Hydroperoxidases (catalases and peroxidases) are ubiquitous “housekeeping” oxidoreductases capable of the heterolytic cleavage of the peroxidic bond predominantly in hydrogen peroxide (H-O-O-H) but also in some small organic peroxides (R-O-O-H. e.g. ethyl hydroperoxide or acetyl hydroperoxide). Especially catalases have the additional striking ability to evolve molecular oxygen (O$_2$) by oxidation of hydrogen peroxide. Thus, enzymes with catalase activity degrade hydrogen peroxide by dismutation.

Several gene families evolved in the ancestral genomes capable of H$_2$O$_2$ dismutation. The most abundant are heme-containing enzymes that are spread among Bacteria, Archaea and Eukarya. They are divided in two main groups, namely typical or “monofunctional” catalases (E.C. 1.11.1.6, hydrogen peroxide – hydrogen peroxide oxidoreductase) and catalase-peroxidases. Both types of heme enzymes exhibit high catalase activities, but have significant differences including absence of any sequence similarity and very different active site, tertiary and quaternary structures. Enzymatic classification of bifunctional catalase-peroxidases is not clear since besides their catalatic activity (E.C. 1.11.1.6) they exhibit a peroxidase activity similar to conventional peroxidases (EC 1.11.1.7, hydrogen peroxide – donor oxidoreductase). Both protein
families are present in prokaryotic and eukaryotic genomes. Non-heme manganese-containing catalases (Mn-catalases) constitute a third (minor) group of catalytically active enzymes. Mn-catalases (E.C. 1.11.1.6), initially referred to as pseudo-catalases, are present only in bacteria. The so far known sequences of all enzymes from these three protein families are collected and annotated in PeroxiBase7 (http://peroxidase.isb-sib.ch).

Catalase versus peroxidase activity
Continuous and effective degradation of H₂O₂ is indispensable for aerobic life8. This is essential for both removal of excessive H₂O₂ and for strict regulation of its concentration in signaling pathways5. As mentioned above nature has developed three protein families that can perform its dismutation at reasonable rates. The overall catalatic reaction includes the degradation of two molecules of hydrogen peroxide to water and molecular oxygen (Reaction 1).

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \quad \text{Reaction 1}
\]

Reaction 1 is the main reaction catalyzed by typical catalases, catalase-peroxidases and Mn-containing catalases. Additionally, several heme-containing proteins, including most peroxidases, metmyoglobin, and methemoglobin have been observed to exhibit a low level of catalatic activity9. From these enzymes multifunctional chloroperoxidase from the ascomycete Caldariomyces fumago has the greatest reactivity as a catalase10. Due to its very limited distribution in nature this protein will not be discussed here in more detail.

In catalases and catalase-peroxidases two distinct stages can be distinguished in the catalatic reaction pathway. The first stage involves oxidation of the heme iron using hydrogen peroxide as substrate to form compound I (Reaction 2)11. The oxygen-oxygen bond in peroxides (R-O-O-H) is cleaved heterolytically with one oxygen leaving as water and the other remaining at heme iron (Reaction 2). Generally, compound I is a redox intermediate two oxidizing equivalents above the resting [i.e. ferric, Fe(III)] state of the enzyme. In monofunctional catalases, compound I is an oxoiron(IV) porphyrin π-cation radical species \([^\cdot]\text{Por Fe(IV)=O}\), which is reduced back to the ferric enzyme by a second molecule of hydrogen peroxide with the release of molecular oxygen and water (Reaction 3). Thus, in a catalase cycle H₂O₂ acts as oxidant (Reaction 2) and reductant (Reaction 3). Compound I can also undergo an intramolecular one-electron reduction with or without a proton resulting in the formation of an alternative compound I (Reaction 4) that is
catalytically inactive. Here, the protein moiety (AA) donates the electron that quenches the porphyril radical. Reaction 4 is responsible for the decrease of catalase activity with time since the formed intermediate returns to the resting enzyme very slowly. A role of NADPH, which binds to some clades of monofunctional catalases, as two-electron donor for the transition of \( ^*\)AA Por Fe(IV)-OH back to ferric catalase has been discussed.

\[
\begin{align*}
\text{Por Fe(III)} + \text{H}_2\text{O}_2 & \rightarrow ^*\text{Por Fe(IV)=O} + \text{H}_2\text{O} & \text{Reaction 2} \\
^*\text{Por Fe(IV)=O} + \text{H}_2\text{O}_2 & \rightarrow \text{Por Fe(III)} + \text{H}_2\text{O} + \text{O}_2 & \text{Reaction 3} \\
\text{AA}^*\text{Por Fe(IV)=O} + \text{H}^+ & \rightarrow ^*\text{AA Por Fe(IV)-OH} & \text{Reaction 4}
\end{align*}
\]

A lively debate exists about the question whether catalase-peroxidases also follow Reactions 2 & 3. As in monofunctional catalases, organic peroxides (e.g. acetyl hydroperoxide) can oxidize catalase-peroxidases to a compound I-like species according to Reaction 2, which is rapidly transformed to a protein radical species\textsuperscript{12,13} with spectral UV-Vis signatures dissimilar to those described in monofunctional catalases\textsuperscript{14}. Thus, in the light of these data a number of alternative reaction schemes for catalase-peroxidases have been proposed, that consider a rapid quenching of the porphyril radical by an electron from the protein moiety. These alternative compound I species have been suggested to be \(^*\)MYW Fe(IV)-OH or more generally \(^*\)AA Fe(IV)-OH, with MYW being the catalase-peroxidase typical distal side covalent adduct (see below) and AA being an alternative amino acid near the heme. In any case, also in catalase-peroxidases the catalatic cycle has two distinct phases with \(\text{H}_2\text{O}_2\) acting as oxidant and reductant. But in contrast to monofunctional catalases this unique compound I species must be able to oxidize \(\text{H}_2\text{O}_2\), i.e. it participates in the catalase cycle.

The mechanism of \(\text{H}_2\text{O}_2\) degradation by non-heme Mn-catalases follows a completely different scheme. The overall reaction (Reaction 1) also holds for these binuclear metalloproteins and again \(\text{H}_2\text{O}_2\) acts as oxidant and reductant. But the mechanism of the two individual reaction steps is completely different.

Catalases and catalase-peroxidases can also catalyze a peroxidatic reaction (Reaction 5) in which electron donors (\(\text{AH}_2\)) are oxidized via one-electron transfers releasing radicals (\(^*\text{AH}\)). Thus, a peroxidase cycle includes three reactions, i.e. compound I formation (Reaction 2), compound I reduction to compound II, an oxoiron(IV) species [PorFe(IV)=O] (Reaction 6) and compound II reduction back to the resting state (Reaction 7). Generally, the peroxidatic reaction of monofunctional catalases is weak but in
bifunctional catalase-peroxidases Reaction 5 is important. Similar to the discussion of the catalase cycle there is also a dispute about the electronic structure of redox intermediates of catalase-peroxidases in the peroxidase cycle\(^{15}\).

\[
\begin{align*}
\text{H}_2\text{O}_2 + 2 \text{AH}_2 & \rightarrow 2 \text{H}_2\text{O} + 2 \cdot \text{AH} & \text{Reaction 5} \\
\text{Por}^*\text{Fe(IV)}=\text{O} + \text{AH}_2 & \rightarrow \text{Por Fe(IV)}=\text{O} + \cdot \text{AH} & \text{Reaction 6} \\
\text{Por Fe(IV)}=\text{O} + \text{AH}_2 & \rightarrow \text{Por Fe(III)} + \text{H}_2\text{O} + \cdot \text{AH} & \text{Reaction 7}
\end{align*}
\]

Peroxidatic reactions can also reduce compound I directly in a two-electron reaction back to the ferric enzyme (Reaction 8). Especially mammalian and yeast catalases have been described to oxidize ethanol or other short-chain aliphatic alcohols to acetaldehyde or corresponding aldehydes\(^8,16\).

\[
\begin{align*}
\text{Por}^*\text{Fe(IV)}=\text{O} + \text{CH}_3\text{CH}_2\text{OH} & \rightarrow \text{Por Fe(III)} + \text{CH}_3\text{CHO} + \text{H}_2\text{O} & \text{Reaction 8}
\end{align*}
\]

**Distribution and phylogeny of catalase-peroxidases**

Bifunctional catalase-peroxidase (KatG) has raised considerable interest, since it represents the only peroxidase with a reasonable high catalatic activity around neutral pH (Reaction 1) besides a usual peroxidase activity (Reaction 6). Its overall fold and active site architecture is typical peroxidase-like, which is underlined by their striking sequence homologies to other members of class I of the plant, fungal and (archae)bacterial heme peroxidase superfamily\(^{17}\). Together with these class I peroxidases (i.e. ascorbate peroxidase and cytochrome \(c\) peroxidase) KatGs have the Pfam accession number PF00141. The InterPro accession number IPR000763 is more specific only for catalase-peroxidase. PeroxiBase currently (August 2008) contains 329 sequences of \(katG\) genes and their evolution was analyzed recently\(^{18}\). The most important output of this investigation is that \(katG\) genes are distributed in about 40% of bacterial genomes and sometimes even closely related species differ in possessing \(katG\) genes of different origin or even do not possess any \(katG\) gene. Two different evolutionary lines of \(katG\) genes also exist in eukaryotes: one in algae and one in fungi and both are expected to originate from lateral gene transfer of corresponding bacterial genomes living in close relationship with the eukaryotes\(^{18,19}\). Phylogenetic analysis reveals that the closest neighbour of all fungal \(katG\) genes is the \(katG\) gene of *Flavobacterium johnsonii*. In fungal KatGs two clades are
found, one encoding cytosolic and the other secreted enzymes. The occurrence of extracellular fungal KatGs is underlined by the prediction of signal sequences.

**Structure(s) and reaction mechanisms of catalase-peroxidases**

The four available crystal structures of the KatGs *Haloarcula marismortui* (1ITK)\textsuperscript{20}, *Burkholderia pseudomallei* (1MWV)\textsuperscript{21}, *Mycobacterium tuberculosis* (1SJ2)\textsuperscript{22} and *Synechococcus* PCC 7942 (1UB2)\textsuperscript{23} revealed that the homodimeric heme \textit{b} enzymes have proximal and distal conserved amino acids at almost identical positions as in other class I peroxidases (Figure 1). In particular both the triads His/Trp/Asp (His279, Trp330 and Asp389; *Burkholderia* numbering) and His/Arg/Trp (His112, Arg108, Trp111) are conserved (Figure 2). Moreover, the proximal His is hydrogen-bonded to the carboxylate side chain of the nearby Asp residue which, in turn, is hydrogen-bonded to the nitrogen atom of the indole group of the nearby Trp residue (Figure 1). However, the X-ray structures also revealed features unique to KatG. In the vicinity of the active site, novel covalent bonds are formed among the side chains of three distal residues including the conserved Trp111 (Figures 1 and 2). In particular, both X-ray crystallization data (Table 1) and mass spectrometric analysis\textsuperscript{24,25} have confirmed the existence of a covalent adduct between Trp111, Tyr238 and Met264 (Figure 1). Exchange of either Trp111 or Tyr238 prevents crosslinking, whereas exchange of Met264 still allowed the autocatalytic covalent bond formation between Trp111 and Tyr238\textsuperscript{25,26}.

In addition to an extra C-terminal copy resulting from gene duplication\textsuperscript{17} that lacks the prosthetic group but supports the architecture of the active site\textsuperscript{27}, other KatG-typical features are three large loops, two of them show highly conserved sequence patterns\textsuperscript{28} and constrict the access channel of H\textsubscript{2}O\textsubscript{2} to the prosthetic heme \textit{b} group at the distal side. The channel is characterized by a pronounced funnel shape and a continuum of water molecules. At the narrowest part of the channel, which is similar but longer and more restricted than that in other monofunctional peroxidases, two highly conserved residues, namely Asp141 (Figures 1 & 2) and Ser324 control the access to distal heme side. Together with a conserved glutamate residue at the entrance both acidic residues seem to be critical for stabilizing the solute matrix and orienting the water dipoles in the channel. Exchange of both residues affects the catalase but not the peroxidase activity. It is interesting to note that in typical catalases similar acidic residues participate in stabilization and orientation of the solute matrix in the main access channel for H\textsubscript{2}O\textsubscript{2}.
Figure 1. Detailed view of active site residues of catalase-peroxidase from *Burkholderia pseudomallei* (*Synechocystis* numbers in parentheses) with the distal active residues Met264, Tyr238 and Try111, which are covalently linked, and the catalytically active His112, Arg108 and Asp141 that contribute to the stabilization of the H-bonding network of the access channel. On the proximal side the conserved amino acid triad His279, Trp330, and Asp389 is depicted. The Figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1MWV).
Figure 2. Selected parts of the multiple sequence alignment of 43 catalase-peroxidases (KatGs). 32 complete fungal sequences are presented together with closely related bacterial counterparts. Similarity scheme is identical with Figure 1. The substitution matrix and the alignment algorithm were the same as used in recent experimental work on this topic. (A) Distal side of the heme group with the catalytic triad. (B) Distal side of heme with important hydrogen bond location. (C) Proximal side of the prosthetic heme group with the essential histidine. (D) Proximal side of heme with important hydrogen bond location. Catalytically important residues are marked with an arrow. Abbreviations for Linnean names and ID numbers correspond to PeroxiBase nomenclature (see http://peroxibase.isb-sib.ch/ for all details).
Figure 2. Continued.
This extensive distal site hydrogen-bonding network causes KatGs to differ from typical peroxidases. Typically, the catalatic but not the peroxidatic activity is very sensitive to mutations that disrupt this network\textsuperscript{29,30}. Moreover, the integrity of this network is crucial for the formation of distinct protein radicals that are formed upon incubation of KatG with peroxides\textsuperscript{13,31}. Mutational analysis clearly underlined the importance of the KatG-typical covalent adduct Trp-Tyr-Met. Its disruption significantly decreased the catalase but not the peroxidase activity. For instance, upon exchange of Tyr238 by Phe the resulting KatG variant completely lost its capacity to oxidize H\textsubscript{2}O\textsubscript{2} and was transformed to a typical peroxidase\textsuperscript{31}. Similarly all other mutations so far performed in the heme cavity or substrate channel of a KatG affected the oxidation and not the reduction reaction of hydrogen peroxide\textsuperscript{15}, which is the initial reaction in all heme hydroperoxidases.

Both $K_M$ and $k_{cat}$ values of KatGs are significant lower compared to typical catalases. Apparent values range from 3,500-6,000 s\textsuperscript{-1} and 3.7-8 mM\textsuperscript{15}. In contrast to typical catalases and Mn-catalases, the catalase activity of KatG has a sharp maximum activity around pH 6.5.

KatG is a bifunctional enzyme. It oxidizes typical artificial peroxidase substrates like $o$-dianisidine, guaiacol or ABTS. The pH profile of the peroxidase activity of KatG has its maximum around pH 5.5 independent of the nature of most donors. Compound I (formed with peroxyacetic acid) reduction by monosubstituted phenols and anilines has been shown to depend on the substitution effect on the benzene ring and follows the Hammet equation\textsuperscript{32} similar to typical peroxidases. Additionally, KatGs have been reported to have also halogenation\textsuperscript{33} and NADH oxidase\textsuperscript{34} activity.

The naturally occurring peroxidase substrate is unknown. In physiological conditions it is well known that KatG from \textit{Mycobacterium tuberculosis} can activate the anti-tuberculosis drug isoniazi4\textsuperscript{15} but otherwise the physiological function of these enzymes remains unknown. Due to the restricted access, only small peroxidase substrates can enter the main entrance channel. A second access route, found in monofunctional peroxidases, approximately in the plane of the heme, is blocked by the KatG-typical loops. However, another potential route that provides access to the core of the protein, between the two domains of the subunit, has been described\textsuperscript{21}. It has been speculated that this could be the binding site for substrates with extended, possibly even polymeric character. In any case neither from prokaryotic nor from eukaryotic KatGs the naturally occurring one-electron donor(s) is known nor is the function of a high peroxidase activity in a catalatic enzyme. A reasonable role at low peroxide concentration could be the reduction of
alternative and catalatically inactive compounds I & II species to ferric KatG (similar to NADPH in clade 3 catalases). Potential role of catalase-peroxidases in H$_2$O$_2$ signaling remains elusive and needs to be investigated on both transcriptional and translational levels. This is an actual topic mainly for eukaryotic catalase-peroxidases as there can exist possible signaling pathways mainly during the interaction between host and pathogen.

Hydrogen peroxide reduction (i.e. compound I formation) follows Reaction 2, which – in contrast to typical catalases – is immediately followed by Reaction 4 forming the catalatically active intermediate $\cdot$AA Por Fe(IV)-OH. One role of the Trp-Tyr-Met adduct could be the (at least transient) radical site ($\cdot$MYW Fe(IV)-OH) that quenches the porphyril radical. Similar to typical peroxidases, compound I formation (i.e. the heterolytic cleavage of H$_2$O$_2$) is clearly assisted by the conserved distal residues His112 and Arg108$^{15}$ but the exact role of distal amino acids in H$_2$O$_2$ oxidation is unknown. The classical compound I, observed in virtually all heme peroxidases and catalases, has a radical located on the porphyrin. However, in some enzymes a migration of an electron from the protein to the heme takes place, thereby quenching the phorphyrin radical and producing a protein radical. Jakopitsch et al.$^{14}$ showed - upon using stopped-flow spectroscopy - that the catalase mechanism in KatG from three different sources (Synechocystis PCC 6803, Burkholderia pseudomallei, and Mycobacterium tuberculosis) diverges from the typical monofunctional enzyme pathway exemplified by bovine liver catalase. Unlike monofunctional catalases, upon addition of millimolar concentrations of H$_2$O$_2$ to ferric KatG was immediately transformed to an intermediate with characteristics of oxyferrous heme$^{14,36,37}$ at pH $\leq$ 6.5, with absorbance maxima at 415 nm, 545 nm and 580 nm, whereas at pH $>$ 7.0 another low-spin species was formed with maxima at 418 nm and 520 nm. These spectral features represent the dominating redox intermediate during H$_2$O$_2$ degradation.

Analysis using stopped-flow spectroscopy in combination with rapid-freeze-quench electronic spin resonance spectroscopy clearly demonstrated the formation of a protein-based radical on the distal side Trp-Tyr-Met adduct that co-exists with hydroxoferryl heme. It persists during peroxide turnover and thus was proposed to be the catalytically competent intermediate in the catalase cycle of wild-type KatG$^{38,39}$. Based on a reaction scheme proposed earlier$^{14}$ and the recent findings by Suarez et al.$^{38}$ it is now possible to propose the catalatic mechanism of KatG (Figure 3).
Figure 3. Reaction scheme of the hydrogen peroxide reduction and oxidation reaction catalyzed by catalase-peroxidase. It summarizes experimental data published by Jakopitsch et al.\textsuperscript{14}, Deemagarn et al.\textsuperscript{40}, Suarez et al.\textsuperscript{38}, and Zhao et al.\textsuperscript{39}.
In detail, the first substrate hydrogen peroxide enters the main channel into the heme site by binding between Arg108 and Asp141 (*Burkholderia pseudomallei* numbering), i.e. one of the two substrate paths proposed by Deemagarn et al.\(^4^0\). Compound I formation is initiated by moving of the H\(_2\)O\(_2\) molecule to interact with Arg108 and His112. During compound I formation two electrons coming from the iron and the porphyrin are transferred to the oxygen, thereby catalyzing the heterolytic cleavage of H-O-O-H and forming an oxoferryl heme with a porphyrin cation radical. This (classical) compound I is rapidly converted to an alternative isoelectronic species with hydroxoferryl heme and the second oxidation equivalent at the Trp-Tyr-Met adduct (Figure 3). The second substrate H\(_2\)O\(_2\) enters the heme cavity on a proposed second path\(^4^0\) between Asp141 and the main chain atoms of Ile237, Tyr238 and Val239 (*Burkholderia pseudomallei* numbering). It finally interacts with the residues Trp111 and His112 and reduces compound I to oxyferrous heme [Fe(II)-O\(_2\) ↔ Fe(III)-O\(_2\)•-], which decomposes to ferric enzyme and superoxide, the latter being quantitatively oxidized at the adduct radical closing the adduct shell and releasing dioxygen\(^1^4,3^8\). This reaction mechanism follows the well known redox interconversion of monofunctional peroxidases in the presence of high concentrations of hydrogen peroxide: ferric peroxidase → compound I → hydroxyferryl → compound III [Fe(II)-O\(_2\) ↔ Fe(III)-O\(_2\)•-] however, the decay of compound III is extremely slow and superoxide is released instead of O\(_2\). In KatG the turnover of the latter reaction is significantly enhanced due to the presence of the adduct radical that also guarantees the release of only dioxygen instead of superoxide.

This reaction scheme is also underlined by the fact that variants that lack the KatG-typical covalent link have lost the catalatic activity. The variants form compound III rapidly, but its turnover [i.e. conversion to Fe(III)] is extremely slow. So there is a clear relationship between the stability of the oxyenzyme form, adduct radical formation and catalase activity\(^3^9\). Of the three distal side mutants, the decay rates of the oxyferrous enzyme was shown to be Met255Ala > Tyr229Phe > Trp107Phe\(^3^9\) (*Mycobacterium* numbering), which is also the order of their catalase activities. Thus, the adduct radical itself or some unique structural feature of this species, or both, contribute to the rapid decay of wild-type oxyenzyme.

In any case, though catalyzing the same overall dismutation reaction (2 H\(_2\)O\(_2\) → 2 H\(_2\)O + O\(_2\)) catalases and catalase-peroxidases follow a different reaction mechanism. As a consequence the catalase activity of KatG should be attributed as pseudo-catalatic.
References


Hydrogen peroxide oxidation by catalase-peroxidase follows a non-scrambling mechanism

Jutta Vlasits, Christa Jakopitsch, Manfred Schwanninger, Peter Holubar and Christian Obinger

Abstract Despite catalyzing the same reaction (2H₂O₂ → 2H₂O + O₂) heme-containing monofunctional catalases and bifunctional catalase-peroxidases (KatGs) do not share sequence or structural similarities raising the question of whether or not the reaction pathways are similar or different. The production of dioxygen from hydrogen peroxide by monofunctional catalases has been shown to be a two-step process involving the redox intermediate compound I which oxidizes H₂O₂ directly to O₂. In order to investigate the origin of O₂ released in KatG mediated H₂O₂ degradation we performed a gas chromatography–mass spectrometry investigation of the evolved O₂ from a 50:50 mixture of H₂¹⁸O₂/H₂¹⁶O₂ solution containing KatGs from Mycobacterium tuberculosis and Synechocystis PCC 6803. The GC–MS analysis clearly demonstrated the formation of ¹⁸O₂ (m/e = 36) and ¹⁶O₂ (m/e = 32) but not ¹⁸O¹⁶O (m/e = 34) in the pH range 5.6–8.5 implying that O₂ is formed by two-electron oxidation without breaking the O–O bond. Also active site variants of Synechocystis KatG with very low catalase but normal or even enhanced peroxidase activity (D152S, H123E, W122F, Y249F and R439A) are shown to oxidize H₂O₂ by a non-scrambling mechanism. The results are discussed with respect to the catalatic mechanism of KatG.

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Keywords: Catalase-peroxidase; Hydrogen peroxide dismutation; Dioxygen release; Gas chromatography–mass spectrometry analysis

1. Introduction

The four available crystal structures of catalase-peroxidases (KatGs) of Halocarcina marismortii (1ITK), Burkholderia pseudomallei (1MWV), Mycobacterium tuberculosis (1SJ2) and Synechococcus PCC 7942 (1UB2) [1–4] revealed that the organization of their active site is similar to those of cytochrome c peroxidase [5] and ascorbate peroxidase [6]. Although belonging to the heme peroxidase superfamily of plant, fungal and bacterial enzymes [7] KatGs [EC 1.11.1.7] catalyze the same reaction as monofunctional catalases [EC 1.11.1.6] namely the dismutation of hydrogen peroxide to dioxygen and water (2H₂O₂ → 2H₂O + O₂). There are no sequence or structural similarities between the two enzyme classes and in contrast to monofunctional catalases the catalatic mechanism of KatGs is under discussion [8,9]. In monofunctional catalases the overall reaction follows two distinct states in the reaction pathway. The first stage involves oxidation of the heme iron using hydrogen peroxide as substrate to form compound I, an oxoiron(IV) porphyril radical (Por⁺Fe⁴⁺=O) species (reaction (1)). The second stage, or reduction of compound I, employs a second molecule of peroxide as electron donor providing two oxidation equivalents (reaction (2)). Gas chromatography–mass spectrometry (GC–MS) analyses have revealed the exclusive formation of ¹⁸O₂ and ¹⁶O₂ from a 50:50 mixture of H₂¹⁸O₂ and H₂¹⁶O₂ indicating that in monofunctional catalasas O₂ is formed by two-electron oxidation of H₂O₂ without breaking the O–O bond [10,11]. Reaction (2) was proposed to include proton abstraction by the distal histidine, hydride-ion transfer to compound I and, finally, liberation of dioxygen and water [8,11]

PorFe³⁺ + H₂O₂ → Por⁺Fe⁴⁺=O + H₂O (1)

Por⁺Fe⁴⁺=O + H₂O₂ → PorFe³⁺ + O₂ + H₂O (2)

In chloroperoxidase the catalase reaction has been shown to proceed also via a non-scrambling mechanism, whereas in a reaction with labeled and unlabeled m-chloroperoxybenzoic acid a scrambling mechanism for the release of O₂ was established [12]. With m-chloroperoxybenzoic acid the oxygen atoms of O₂ derived from different molecules suggesting that at least one oxygen atom came from compound I (Por⁺Fe⁴⁺=O) [12]. A clear picture of the reaction pathway of KatG has remained elusive. The determination of crystal structures of KatGs [1–4] has led to the identification of several KatG–specific residues, subsequently confirmed by site-directed mutagenesis studies [9]. Such features have suggested a number of unusual mechanisms controlling the catalase reaction. Moreover, the spectroscopic signatures of the redox intermediates are different from monofunctional catalases and the exact electronic nature of the redox intermediate in the catalatic cycle is unknown [9]. Thus, in order to investigate whether KatG mediates the direct two-electron oxidation of H₂O₂ to O₂ a GC–MS analysis was performed using ¹⁶O- and ¹⁸O-labeled H₂O₂. Catalase-peroxidases from two different organisms (Mycobacterium...
tuberculosis and Synechocystis PCC 6803) and the most interesting Synechocystis KatG variants with diminished catalatic activity have been investigated. For comparison, the enzymatic action of bovine liver catalase (BLC, representative of monofunctional catalases) was elucidated under identical assay conditions. The results in all cases unambiguously show that the O₂ evolved originates from intact O-O bonds in hydrogen peroxide. The catalatic mechanism of KatG is discussed.

2. Materials and methods

2.1. Materials

Concentrations of H₂O₂, obtained as a 30% solution from Sigma, and H₂¹⁵O₂ (90% ¹⁵O), obtained as a 2% solution from ICON ISOTOPES, were determined by using a molar extinction coefficient of Ɛ₃⁴⁰ = 39.4 M⁻¹ cm⁻¹. Additionally, the concentration of H₂O₂ was determined titrimetrically by oxidation with KMnO₄ in acified solution according to 2MnO₄⁻ + 5H₂O₂ + 2H⁺ → 2Mn²⁺ + 5O₂↑ + 8H₂O with 1 ml KMnO₄ (0.002 mol l⁻¹) corresponding to 34.02 µg H₂O₂ [13]. Titrisol® potassium permanganate solution, [KMnO₄] = 0.02 mol l⁻¹ was obtained from Merck. The 2 ml screw top vials with septa, Viton® were obtained from Supelco. Xanthine oxidase from buttermilk, xanthine, Cu-Zn superoxide dismutase from bovine erythrocytes, horse heart cytochrome c and nitro blue tetrazolium chloride (NBT) were obtained from Sigma.

Bovine liver catalase (BLC) was obtained from Sigma and the concentration was calculated using the extinction coefficient of Ɛ₃⁴⁰ = 1.2 × 10⁶ M⁻¹ cm⁻¹. Recombinant catalase-peroxidase from Synechocystis and Mycobacterium tuberculosis were produced in E. coli and purified as previously reported [14,19]. The concentrations of Synechocystis and M. tuberculosis catalase-peroxidase (KatG) were calculated from a molar extinction coefficient of Ɛ₃⁴⁰ = 1.0 × 10⁶ M⁻¹ cm⁻¹. Mutant expression and purification of the Synechocystis KatG variants Y249F, W122F, H123E, D152S and R439A were described previously [15–19].

2.2. Sample preparation

All solutions (total volume: 100 µl) and vials used in the assays were made oxygen-free by flushing with nitrogen gas (<3 ppm) and storing in a glove box (Meca-Plex, Neugebauer) with a positive pressure of nitrogen (25 mbar).

Hydrogen peroxide stock solutions were mixed with 100 mM phosphate/citrate buffer (pH 5.6) and hydrogen peroxide in 100 mM phosphate/citrate buffer (pH 5.6–8.5) containing 100 mM – 200 mM) dismutated by BLC. Furthermore, quantification of dioxygen released by enzymatic activity was compared to that produced when H₂O₂ was oxidized by a small excess of KMnO₄. The amount of KMnO₄ that was required for complete oxidation of hydrogen peroxide was judged by getting the acidic solution to turn slightly violet.

2.4. Polargraphic measurements

Dioxygen release from H₂O₂ (1–10 mM) was determined polargraphically using a Clark-type electrode (YSI 5301 oxygen probe) inserted into a stirred thermostated water bath (YSI 5301B). To cover the pH range 5.6–8.5, 50 mM phosphate/citrate, 50 mM phosphate or 50 mM Tris–HCl buffers containing 100 µM ethylene-diamine-tetra-acetic acid (EDTA) were used. All reactions were performed at 30 °C, initiated by addition of KatG and performed in the presence and absence of superoxide dismutase (10 µg/ml).

3. Results and discussion

One method of approach to the study of enzymes with catalatic activity is to focus on their reaction product molecular oxygen. Usually the enzymatic activity is followed polargraphically and the turnover numbers given in Table 1 are based on such measurements using a Clark-type electrode [8,14–19]. Table 1 shows that the kcat value of the monofunctional catalase BLC is about two orders of magnitude higher than that of KatGs. Nevertheless, KatGs are the only heme peroxidases from the superfamily of plant, fungal and bacterial enzymes, which exhibit peroxidase and substantial catalase activities similar to monofunctional catalases (Table 1). But the catalase activity is poorly characterized and it has been unknown so far whether the two O-atoms in each O₂ liberated came from the same H₂O₂ molecule or from two separate H₂O₂ molecules.

Precondition was a clear separation of the main components of air, N₂ (78.084 v/v%) and O₂ (20.946 v/v%). In Fig. 1A it is shown that the MoleSieve capillary column effectively separated O₂ (2.03 min) from N₂ (2.7 min). Analysis of the headspace of a reaction mixture containing 100 mM H₂O₂ in 100 mM phosphate buffer, pH 7.0, prepared in the glove box and in the absence of an enzyme, gave a dominating N₂-peak and about 0.5–0.8% O₂ most probably deriving from syringe discharge from the glove box, transport to and injection onto the gas chromatograph (thin line). The change in relative peak areas upon total dismutation of 100 mM H₂O₂ by 10 µM catalase-peroxidase from Synechocystis (SynKatG) (sampling of
Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>kcat (s⁻¹)</th>
<th>mlz 32 (¹⁶O₂) (% O₂)</th>
<th>mlz 36 (¹⁸O₂) (% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLC</td>
<td>212000</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>MtbKatG</td>
<td>6000</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>SynKatG</td>
<td>3500</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>D152S</td>
<td>200</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>H123E</td>
<td>2</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>W122F</td>
<td>6</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>Y249F</td>
<td>170</td>
<td>46</td>
<td>54</td>
</tr>
</tbody>
</table>

The enzymes were incubated with 50 mM H₂¹⁸O₂ and 50 mM H₂¹⁶O₂ in 100 mM phosphate buffer, pH 7.0, for 15 min in the glove box. Enzyme concentrations used for GC–MS analyses: 10 μM (BLC, MtbKatG, SynKatG), 20 μM (D152S), 40 μM (R439A and Y249F), and 80 μM (W122F and H123E), respectively. 20 μl of head-space were analyzed by GC–MS as described in Section 2. Areas of selected ion chromatograms m/z = 32 and m/z = 36 are given in percentage of total oxygen concentration [100% = (m/z = 32) + (m/z = 34) + (m/z = 36)]. All experiments were repeated at least three times and the mean values are given. Average errors on m/z = 32 and m/z = 36 values are ±5%.

head-space 15 min after addition of SynKatG) is evident (Fig. 1A, bold line). The release of 50 mM O₂ from 100 mM H₂O₂ led to a significant increase of O₂ concentration in the head-space as demonstrated by the significant increase in peak area at 2.03 min. Independent of the mechanism of O₂ release from H₂O₂ (enzymatic dismutation or chemical oxidation by KMnO₄) there was a linear correlation between the H₂O₂ concentration in the assay and the calculated peak area at 2.03 min (inset to Fig. 1A). With the exception of the variants W122F and H123E, under the substrate to protein ratios used in these assays enzyme inactivation could be disregarded [14] and H₂O₂ was completely degraded before sampling.

Fig. 1B shows the mass analysis in the SIM mode of the 2.03 min peak derived from sampling the head-space 15 min after addition of 10 μM SynKatG to a mixture of 50 mM H₁⁶O₂ and 50 mM H₁⁸O₂ in 100 mM phosphate buffer, pH 7.0. Very similar results within experimental error were obtained with MtbKatG (10 μM) and BLC (10 μM). In any case, two peaks of equal areas for H₂⁰O₂ (m/z = 36) and H₂O₂ (m/z = 32) with no indication of O²⁻¹⁸O₂ (m/z = 34) formation were found. This clearly demonstrates that O₂ is formed by two-electron oxidation of H₂O₂ without breaking the O–O bond. In this respect there is no difference between KatG and monofunctional catalases.

The pH profiles of catalatic activity for KatGs are quite different from those for monofunctional catalases. The latter’s catalatic activities are essentially pH-independent from pH 5 to 10 [20], whereas KatGs show a sharp optimum between pH 6 and 7 [9]. Performing the assays described above at pH 5.6 and 8.5 and analyzing the head-space by GC–MS gave very similar results as shown in Fig. 1B indicating that the H₂O₂ oxidation follows the retention mechanism in the whole activity range of KatG. Thus, the drop in activity both at acidic and alkaline pH must be due to other mechanisms. These could include proton abstraction before oxidation of H₂O₂ with the help of an acid–base catalyst at the distal heme site and/or protonation of the active compound I species and/or pH-induced changes in its electronic structure (see below).

Analysis of the crystal structures [1–4] and mass spectrometric data [21,22] has demonstrated the presence of two covalent bonds between three amino acid side chains, W122, Y249, and M275 (Synechocystis numbering). This M–Y–W crosslink appears to be a characteristic common to all KatGs and has been demonstrated to be essential for the catalase activity [9,15,16,19]. Interestingly, this adduct can be associated with a KatG-typical arginine (R439) [2]. Its association with the tyrosinate ion on the adduct is also required for optimum catalatic rates [19,23]. Another important residue at the distal heme cavity is D152, which is part of the substrate channel and has its side chain carboxyl group pointing toward the heme pocket. It participates in maintaining a rigid and
extended hydrogen-bond network, which is important for the H$_2$O$_2$ oxidation reaction [18,24]. Variants that had these residues exchanged (D152S, W122F, Y249F, and R439A) showed a significantly reduced catalase (see Table 1) but a normal or even enhanced peroxidase activity [9]. Thus, we included these variants in this mechanistic investigation. Additionally, H123E was probed since the distal H123 belongs to the very few amino acids, which are important in both the peroxidase and catalase activity because of its function in compound I formation [17]. All these KatG variants are interesting since they exhibit an altered kinetics of interconversion as well as spectral signatures of redox intermediates. This indicates that the electronic nature of the active compound I species might be different as could be the mechanism of H$_2$O$_2$ oxidation [9].

In order to see whether these variants also follow the retention mechanism, the dismutation of a 50:50 mixture of labeled and unlabeled H$_2$O$_2$ was performed and the isotopic distribution of the reaction product O$_2$ was analyzed by mass spectrometry. Higher enzyme concentrations were used (Table 1) because of the diminished turnover. With the exception of H123E and W122F in all assays the substrate was degraded completely within 15 min of reaction. In case of H123E and W122F about 55% and 65% of H$_2$O$_2$ were metabolized as judged from the total 2.03 min O$_2$ peak area. Nevertheless, as Table 1 shows it is evident that with all these variants the oxygen atoms of O$_2$ derive from the same substrate molecule. Although in some cases the catalatic activity is extremely low and the variant is known to behave like a monofunctional peroxidase (e.g. Y249F) the mechanism of H$_2$O$_2$ dismutation follows the non-scrambling mechanism as in the wild-type enzyme.

It has to be mentioned that a non-scrambling mechanism could also result from one-electron oxidation steps of hydrogen peroxide by compound I and/or compound II. In this alternative mechanism superoxide would be a reaction product that dismutates to dioxygen and hydrogen peroxide in a pH-dependent manner. If superoxide is a product in the degradation of hydrogen peroxide by catalase-peroxidase, its dismutation would not necessarily be rate limiting. Nevertheless, this putative pathway can be rejected for several reasons. Firstly, during H$_2$O$_2$ degradation by KatG neither cytochrome c nor NBT reduction was observed (not shown) and secondly, superoxide dismutase (SOD) did not affect the kinetics of oxygen release mediated by KatG (not shown).

From the above experiments we deduce that catalase-peroxidases oxidize H$_2$O$_2$ in a two-electron oxidation step with both oxygen atoms deriving from the same hydrogen peroxide molecule. This non-scrambling mechanism is independent of pH and is not affected by manipulation of highly-conserved and important catalatic residues. Principally, there are two possible mechanisms of the formation of O$_2$ following this retention mechanism. (A) An ionic mechanism via initial proton abstraction with the help of an acid–base catalyst followed by a hydride-ion removal from H$_2$O$_2$ and release of O$_2$. (B) A hydrogen atom transfer from H$_2$O$_2$ to the ferryl species to yield a radical intermediate as has been proposed for the alkaline hydroxylation by cytochrome P450 [25]. Based on experiments with monofunctional catalases and myoglobin variants and deuterium isotope effects on the catalatic activities it has been shown that the ionic mechanism is followed when a general acid–base catalyst is available at the distal cavity at a proper position [11]. In case of KatG two distal side residues have been proposed to function as proton acceptor of the second H$_2$O$_2$ molecule, namely W122 [16] and D152 [18,26]. Not clear is the electronic nature of KatG compound I that is active in H$_2$O$_2$ oxidation. The differences in the spectral features of reaction intermediates formed during H$_2$O$_2$ dismutation by monofunctional catalases and KatGs are significant [8,9]. In monofunctional catalases the classical compound I is active (i.e. oxoiron(IV)porphyril radical) whereas in KatG – due to electron transfer from the protein to the iron – alternative compound I species (e.g. oxoiron(IV) protein radical or even doubly oxidized protein species) have been suggested to be catalatically active [8,9]. In any case, the present investigation unequivocally suggests, that the non-scrambling mechanism is followed generally by catalatic active heme enzymes and seems to be independent of the actual electronic structure of compound I.

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References


Trapping the redox intermediates in the catalase cycle of catalase-peroxidases from *Synechocystis* PCC 6803, *Burkholderia pseudomallei*, and *Mycobacterium tuberculosis*

Christa Jakopitsch, Jutta Vlasits, Ben Wiseman, Peter C. Loewen and Christian Obinger

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Redox Intermediates in the Catalase Cycle of Catalase-Peroxidases from Synechocystis PCC 6803, Burkholderia pseudomallei, and Mycobacterium tuberculosis

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ABSTRACT: Monofunctional catalases (EC 1.11.1.6) and catalase-peroxidases (KatGs, EC 1.11.1.7) have neither sequence nor structural homology, but both catalyze the dismutation of hydrogen peroxide (2H2O2 → 2H2O + O2). In monofunctional catalases, the catalatic mechanism is well-characterized with conventional compound I [oxoiron(IV) porphyrin π-cation radical intermediate] being responsible for hydrogen peroxide oxidation. The reaction pathway in KatGs is not as clearly defined, and a comprehensive rapid kinetic and spectral analysis of the reactions of KatGs from three different sources (Synechocystis PCC 6803, Burkholderia pseudomallei, and Mycobacterium tuberculosis) with peroxyacetic acid and hydrogen peroxide has focused on the pathway. Independent of KatG, but dependent on pH, two low-spin forms dominated in the catalase cycle with absorbance maxima at 415, 545, and 580 nm at low pH and 418 and 520 nm at high pH. By contrast, oxidation of KatGs with peroxyacetic acid resulted in intermediates with different spectral features that also differed among the three KatGs. Following the rate of H2O2 degradation by stopped-flow allowed the linking of reaction intermediate species with substrate availability to confirm which species were actually present during the catalase cycle. Possible reaction intermediates involved in H2O2 dismutation by KatG are discussed.

All aerobically growing organisms have to deal with reactive oxygen species (ROSs), e.g., superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Nature has evolved specialized enzymes for the degradation of the different ROSs to protect the cells. The obvious role of catalases is to dismutate hydrogen peroxide into water and oxygen. There is a high degree of diversity among catalatically active enzymes, and according to their primary and quaternary structure, subunit size, and prosthetic group, they have been divided into four subgroups: monofunctional heme b- or d-containing catalases, bifunctional heme b-containing catalase-peroxidases, nonheme catalases, and, finally, proteins with minor catalatic activity like monofunctional peroxidases (1). Here, we focus on the heme b-containing monofunctional catalases and catalase-peroxidases (KatGs).† Monofunctional catalases are found in all kingdoms of life, whereas KatGs are found only in archaea, bacteria, and fungi (2).

Although both types of heme enzymes exhibit high catalatic activities, there are significant differences, including the absence of any sequence homology and very different tertiary and quaternary structures, including the active site residues (Figure 1). The most highly conserved part in catalases is an eight-stranded antiparallel β-barrel domain with six α-helical insertions in the turns between the strands. The internal parts of this domain harbor essential distal side residues His74, Ser113, and Asn147 (BLC numbering) and the proximal heme iron ligand Tyr357 (3). In contrast, KatGs contain 20 α-helices per monomer, 10 in the N-terminal domain and 10 in the C-terminal domain organized in a manner very similar to that of other class I peroxidases (4, 5). Heme is bound only to the N-terminal domain, and the function of the duplicated C-terminal domain is still under discussion (6). The active side residues include Arg108, His112, and Trp111 (BpKatG numbering) on the distal side of the heme and the proximal ligand His279 (Figure 1D). A covalent adduct involving Trp111, Tyr238 [situated on a loop not found in the other class I peroxidases but highly conserved in KatGs (7)], and Met264 has been shown to be essential for the catalatic activity of KatGs (8−13). Catalase or peroxidase cycles are initiated by the H2O2-mediated oxidation of the native ferric enzyme to com-

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1 Abbreviations: KatG, catalase-peroxidase; SynKatG, catalase-peroxidase from Synechocystis PCC 6803; BpKatG, catalase-peroxidase from B. pseudomallei; MtbKatG, catalase-peroxidase from M. tuberculosis; WT, wild-type; CCP, cytochrome c peroxidase; BLC, bovine liver catalase; PAA, peroxyacetic acid; mCPB, m-chloroperbenzoic acid; CT, charge transfer; EPR, electronic paramagnetic resonance; QM/MM, quantum mechanical/molecular mechanical.

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Generally, compound I is a redox intermediate two oxidizing equivalents above the resting state. This reaction causes the release of one water molecule and coordination of the second oxygen atom to the iron center \( \text{Por}^{\text{IV}} \text{Fe}^{\text{III}}=\text{O} \) \((16)\). In monofunctional catalases, compound I is an oxoiron(IV) porphyrin \( \pi \)-cation radical species \( \text{Por}^{\text{IV}} \text{Fe}^{\text{III}}=\text{O} \) \((16)\) which is reduced back to the ferric enzyme by a second molecule of hydrogen peroxide with the release of oxygen and water \((14)\). In the reaction of BLC with peroxoacetic acid, a tyrosine radical can be formed by migration of an electron to the porphyrin \( \pi \)-cation radical. However, the high rate of turnover suggests that the tyrosyl radical plays no role in the catalatic reaction \((16)\).

A lively debate about the catalatic mechanism in KatGs continues. Like that of BLC, reaction of KatGs with organic peroxides generates an oxoiron(IV) porphyrin \( \pi \)-cation radical species, which rapidly transforms to a protein radical species \((17-19)\), but with spectral UV–vis signatures dissimilar to those described in monofunctional catalases and peroxidases \((1, 8-11)\). Because of the high intrinsic catalase activity generating molecular oxygen, it has been difficult to trap the spectroscopic signatures of the \( \text{H}_2 \text{O}_2 \)-generated catalatic intermediates. In this paper, we have used stopped-flow techniques to characterize the UV–vis signatures of the dominant catalatic intermediates of KatGs from three different sources \((\text{Synechocystis PCC 6803, Mycobacterium tuberculosis, and Burkholderia pseudomallei})\) at pH 5.6, 7.0, and 8.5 and to monitor \( \text{H}_2 \text{O}_2 \) degradation under identical conditions. So far, almost all mechanistic studies have been conducted with these three enzymes, which share all KatG-typical structural features but also showed some differences in radical transformation when treated with organic peroxides \((17-19)\). Significant differences in comparison to BLC are documented and discussed in terms of possible reaction schemes.

**MATERIALS AND METHODS**

Reagents. Standard chemicals and biochemicals of the highest available grade were obtained from Sigma. Hydrogen peroxide was from Sigma, and its concentration was deter-
For comparison with KatGs, we revisited BLC to monitor the spectral features of intermediates formed during the reaction of the ferric enzyme with H$_2$O$_2$ and the kinetics of H$_2$O$_2$ degradation at 240 nm in the stopped-flow apparatus. As an example, a 2 μM solution of BLC depleted a 2 mM solution of H$_2$O$_2$ within 300 ms (Figure 2D). The time trace of H$_2$O$_2$ depletion can be exactly represented by a single-exponential equation (inset of Figure 2D). The spectral features that predominated during the reaction strongly resembled those of the ferric enzyme, consistent with a catalytic cycle of only reactions 1 and 2 where $k_2 > k_1$, as described in the literature (1). No red shift of the Soret band.
or other features typical of a BLC compound I species were observed over the pH range of 5–10 (not shown).

In BLC and monofunctional heme b catalases, generally an oxoiron(IV) porphyril radical compound I (characterized by a hypochromicity at the Soret region of 40–45% and a long wavelength band at 665–670 nm of an intensity almost equal to that of the original CT band of the ferric enzyme) is formed during reaction with peroxoacetic acid (1) (Figure 2A, spectrum 3). The reaction of preformed compound I with hydrogen peroxide (reaction 2) was followed in sequential-mixing mode, revealing that BLC compound I readily reacts with hydrogen peroxide directly to the ferric enzyme (Figure 2A), underlining the fact that the oxoiron(IV) porphyril radical species indeed participates in the catalytic cycle and is responsible for the oxidation of H₂O₂ to O₂. Spectra 1 and 2 in Figure 2 recorded 1.3 and 20 ms, respectively, after mixing 10 μM H₂O₂ with 3 μM BLC compound I already resemble (15–20% hypochromicity) that of the ferric enzyme and did not change during H₂O₂ degradation. After complete H₂O₂ dismutation, the compound I spectrum reappeared as a result of reaction with excess peroxoacetic acid (spectrum 3, Figure 2). The time trace for reduction of compound I by hydrogen peroxide was fitted to a single-exponential equation, and the resulting linear plot of the pseudo-first-order rate constants versus H₂O₂ concentration (Figure 2C) produced a second-order rate constant (k₂) of 3.2 × 10⁷ M⁻¹ s⁻¹ at pH 7.0, in good agreement with the value estimated by Chance (21).

Kinetics of Hydrogen Peroxide Degradation Mediated by Catalase-Peroxidases. A significant difference in the turnover rates of BLC and KatG is evident (compare Figures 2D and 3C), with 2 μM KatG from Synechocystis (SynKatG) degrading 10 mM hydrogen peroxide in ~1.4 s compared to 0.5 s for 2 μM BLC. Furthermore, the kinetics of degradation of H₂O₂ by KatG differed from those of BLC in that they could not be fitted to a single-exponential equation (Figure 3B–D). Similar results were obtained with KatGs from M. tuberculosis and B. pseudomallei.

To understand the differences in the shape of the time traces between BLC and KatG, it is advisable to describe the H₂O₂ dismutation reaction by a bi-univalent mechanism similar to the reaction catalyzed by superoxide dismutase, SOD (i.e., dismutation of superoxide to hydrogen peroxide and oxygen). According to the mechanism described for SOD by Fee and Bull (22), the catalatic cycle could be described by two irreversible (an oxidative and a reductive) reactions, with E being the enzyme in its resting state and CI representing a compound I species irrespective of its electronic structure:

\[
E + H₂O₂ \overset{k₁}{\underset{k⁻₁}{\rightleftharpoons}} [E-H₂O₂] \overset{k₂}{\rightarrow} CI + H₂O \quad (3)
\]

\[
CI + H₂O₂ \overset{k₃}{\underset{k⁻₃}{\rightarrow}} [CI-H₂O₂] \overset{k₄}{\rightarrow} E + O₂ + H₂O \quad (4)
\]

The steady-state equation can be obtained using the method of King and Altman as follows (23):

\[
-\frac{d[H₂O₂]}{dt} = \frac{2(1/k₂ + 1/k₄)^{-1}[catalase][H₂O₂]}{(1/k₂ + 1/k₄)^{-1} + [H₂O₂]}
\]

The productive binding rates for the oxidative (k₀) and reductive (k₁) half-reactions can be formulated as

\[
k₀ = k₁[k₂(k₋₁ + k₂)]
\]

\[
k₁ = k₃[k₄(k₋₃ + k₄)]
\]

Substitution of these binding rates into the steady-state equation allows the expression of the turnover number (kₜₐₜ) and the apparent Michaelis–Menten constant, Kₘ:

\[
kₜₐₜ = \frac{(1/k₂ + 1/k₄)^{-1}}{(1/k₀ + 1/k₁)^{-1}}
\]

This leads to two limiting cases. (A) A Kₘ much greater than the H₂O₂ concentration corresponds to a second-order process and represents the binding of hydrogen peroxide. A pseudo-first-order degradation rate of H₂O₂ will be monitored at 240 nm. (B) A Kₘ much lower than the H₂O₂ concentration represents saturation of the enzyme. A zero-order degradation rate of H₂O₂ will be monitored at 240 nm.

An apparent Kₘ value for BLC has been determined to be 93 mM (24), but our studies are limited to concentrations of hydrogen peroxide up to only 20 mM, because of the increasing inaccuracy at absorbances in excess of 1 and the vigorous oxygen evolution in the reaction cell at higher concentrations. Therefore, in the case of BLC where Kₘ >> [H₂O₂], a pseudo-first-order reaction is expected and observed (Figure 2D). By contrast, the Kₘ values of KatGs are much lower [4.2 mM for SynKatG, 2.5 mM for MtKatG (8), and 5.9 mM for BpKatG (25), all at pH 7]. As a consequence, the shape of the time traces depends on the H₂O₂ concentration, following almost pseudo-first-order kinetics at H₂O₂ concentrations below 1 mM (Figure 3A) and deviating from the single-exponential fit at 10 mM H₂O₂ (Figure 3B). At alkaline pH, however, the rate of depletion of H₂O₂ was nearly linear at low concentrations of hydrogen peroxide, and the apparent Kₘ values at pH 8.5 were determined to be 0.4 mM (SynKatG) at 30 °C and 0.22 mM (BpKatG) at 37 °C, fully compatible with the observed linearity of the time traces and the kinetic model proposed above. At pH 8.5 and 10 mM H₂O₂, it follows that Kₘ ≪ [H₂O₂], and the model predicts a reaction that follows zero-order kinetics, which is reflected by the experimental findings (Figure 3C). At acidic pH, the apparent Kₘ values are comparable to those determined at pH 7.0 (4.7 mM for SynKatG and 5.7 mM for BpKatG at pH 5.6) and the time traces for 10 mM H₂O₂ degradation were nonlinear (Figure 3D) and did not fit well to a single-exponential equation. The plot of the rate of H₂O₂ degradation determined by stopped-flow against pH was similar in shape to a plot using rates obtained from polarographic measurements of oxygen.
A recently constructed variant of SynKatG, E253Q Peroxide, shown. Catalatic Intermediates of Catalase-Peroxidase

Evolution (Figure 3E,F), but with maximum activity slightly shifted from pH 6.5 to 6.0. Similar results were obtained with MtbKatG and BpKatG, although the decrease in stopped-flow-determined activity in the acidic region was much more pronounced than in the polarographically determined rates. These small differences notwithstanding, the pH profiles of all KatGs exhibit a sharp optimum at pH 6–6.5, whereas BLC, and catalases in general, exhibit a broad pH optimum extending from pH 5.6 to 8.5 (not shown).

Reaction of Ferric Synechocystis KatG with Hydrogen Peroxide. A recently constructed variant of SynKatG, E253Q situated in the substrate entrance channel, has been found to slow the catalatic turnover sufficiently to allow the identification of spectral features of reaction intermediates in the catalase cycle (26). With a rate too rapid to be measured by stopped-flow techniques, a 50-fold excess of H2O2 generated an intermediate exhibiting the spectral features of a low-spin species, including a red-shifted Soret band, hyperchromicity in the Q-band region (502 and 542 nm), formation of a broad shoulder around 520 nm, and disappearance of the high-spin CT band at 637 nm. Carrying out the same experiment with native SynKatG produced an intermediate with the same spectral features, but only at a much higher excess (at least 1000-fold) of H2O2, necessitated by the higher catalatic activity of native KatG compared to E253Q (Figure 4). Generally, increasing amounts of H2O2 caused an increasingly more pronounced red shift of the Soret band within 1.3 ms of mixing, and the reaction intermediate was evident only until all of the H2O2 was exhausted (inset of Figure 4). For example, after 1 s, the time needed to completely deplete 10 mM H2O2 with 2 µM SynKatG, the spectrum of ferric protein was recovered.

The pH dependence of the appearance of the reaction intermediate was directly related to the pH dependence of the catalatic reaction. At pH 8.5, the reaction is only 15% of the rate at pH 7, resulting in less H2O2 being necessary for the appearance of the spectral signatures of the intermediate (Figure 5A). No other spectral changes were evident even with a 1000-fold excess of H2O2 at pH 8.5 (Figure 5A). A different situation was observed at acidic pH (Figure 5B) where the first intermediate that could be trapped had spectral features completely different from those observed at pH 7.0 and 8.5. A 1000-fold excess of H2O2 within 1.3 ms generated a spectrum with a slightly decreased and red-shifted Soret band, and addition of even greater excesses (up to 100000-fold) caused a shift of the Soret band to 417 nm and the appearance of two distinct peaks at 545 and 578 nm (Figure 6A,B). As the insets of panels A–C of Figure 5 indicate, there was a pH-dependent correlation between H2O2 degradation and the intermediate present. Analysis of spectra at pH 8.5 and 5.6 (Figure 6A,B)
in conjunction with the rates of H$_2$O$_2$ degradation (Figure 6C,D) reveals a single spectral signature (418 and 520 nm) at pH 8.5 throughout the H$_2$O$_2$ degradation phase and continuously changing spectra during advanced H$_2$O$_2$ depletion at pH 5.6 starting with a 1.3 ms spectrum with peaks at 415, 545, and 580 nm and ending with the Soret band at 407 nm and reappearance of the CT band at 637 nm.

**Reaction of SynKatG Sequentially with Peroxoacetic Acid and H$_2$O$_2$.** The oxidation of KatGs by peroxyacetic acid has been shown to give rise to a calculated reaction rate of $2-6 \times 10^4$ M$^{-1}$ s$^{-1}$, depending on the source of KatG (8, 11, 29). The spectral signatures of the SynKatG compound I intermediate include a 40–50% hypochromicity of the Soret band and two distinct bands at 604 and 643 nm which were attributed to an oxoiron(IV) porphyrin radical cation species.

**Figure 5:** pH dependence of the reaction of ferric wild-type Synechocystis KatG with hydrogen peroxide. (A) Spectra recorded 1.3 ms after 2 $\mu$M ferric KatG (gray line) was mixed with 200 $\mu$M (thin line) and 2 mM (thick line) hydrogen peroxide at pH 8.5. Conditions: 50 mM phosphate buffer at pH 8.5 and 25 °C. The inset shows time traces at 240 nm (black line) and 521 nm (gray line) for the reaction of 2 $\mu$M wild-type KatG with 2 mM hydrogen peroxide at pH 8.5. (B) Spectra recorded 1.3 ms after 2 $\mu$M ferric KatG (gray line) was mixed with 2 mM (thin line) and 200 mM (thick line) hydrogen peroxide at pH 5.6. Conditions: 50 mM phosphate buffer at pH 5.6 and 25 °C. The inset shows time traces at 240 nm (black line) and 429 nm (gray line) for the reaction of 2 $\mu$M wild-type KatG with 10 mM hydrogen peroxide at pH 5.6. Conditions as in panel B. (C) Spectra recorded 1.3 ms after 3 $\mu$M ferric KatG (gray line) was mixed with 10 mM (thick line) hydrogen peroxide at pH 6.5. Conditions: 50 mM phosphate buffer at pH 6.5 and 25 °C. The inset shows time traces at 240 nm (black line) and 429 nm (gray line) for the reaction of 2 $\mu$M wild-type KatG with 10 mM hydrogen peroxide at pH 6.5. Conditions as in panel C.

In contrast to BLC, the compound I form of SynKatG produced by PAA did not appear to react readily with moderate levels of H$_2$O$_2$ back to the resting state. However, the addition of a large excess of hydrogen peroxide did cause a spectral transition, suggesting formation of the same intermediates that were formed in the direct reaction of ferric SynKatG with H$_2$O$_2$ (Figure 7A). For example, spectra recorded during the reaction of 3 $\mu$M SynKatG compound I with 6 mM hydrogen peroxide at pH 6.5 reveal a reaction intermediate with a Soret band at 418 nm, a broad shoulder at 520 nm, and no absorbance in the CT region while H$_2$O$_2$ degradation was taking place. Upon depletion of H$_2$O$_2$, the PAA-generated compound I reappeared, a result of the excess.

**Figure 6:** pH dependence of spectral transitions during hydrogen peroxide turnover of Synechocystis KatG. (A) Reaction of 2 $\mu$M KatG with 2 mM H$_2$O$_2$ at pH 8.5. The thick line is the spectrum 1.3 ms after mixing. This spectral signature persisted for 1.5 s. Subsequent spectra were taken after 1.8 s and after 2.2 s. For orientation, the spectrum of ferric KatG at pH 8.5 is colored gray. (B) Reaction of 2 $\mu$M KatG with 20 mM H$_2$O$_2$ at pH 5.6. The thick line is the spectrum observed 1.3 ms after mixing. Subsequent spectra were taken at 430 ms and 1.4 s. The gray line is the spectrum for ferric KatG at pH 5.6. (C) Hydrogen peroxide depletion recorded at 240 nm for the reaction in panel A. (D) Hydrogen peroxide depletion recorded at 240 nm for the reaction in panel B.

(20). No pH-dependent differences in the kinetics of its formation or its spectral properties were observed in the pH range of 5.6—8.5. EPR has demonstrated that upon reaction of SynKatG with peroxyacetic acid in the absence of electron donors, a Por$^+$ and, subsequently, two protein-based radicals, a Trp$^*$ and a Tyr$^*$, are formed (10).
PAA being in the reaction mixture (not shown). The time course of this reaction as reflected by hyperchromicity and a shift to 418 nm of the Soret band during the first 50 ms was monophasic (black line in Figure 7C) and could be fit to a single-exponential equation (gray line). Plotting these pseudo-first-order rate constants versus H$_2$O$_2$ concentration (inset of Figure 7C) yielded a second-order rate constant of $1.3 \times 10^4$ M$^{-1}$ s$^{-1}$. The intercept of the plot was relatively high (38 s$^{-1}$) which reflected the fact that the formed intermediate was not a stable end product but subjected to permanent turnover during H$_2$O$_2$ degradation. At pH 8.5, the spectral transitions were similar to those at pH 7.0 (not shown), whereas at pH 6.0, the Soret band shifted to 416 nm which was accompanied by absorbance decreases at 604 and 643 nm and the appearance of peaks at 545 and 580 nm all following monophasic kinetics with a calculated rate constant of $1.5 \times 10^4$ M$^{-1}$ s$^{-1}$ (intercept of 12 s$^{-1}$) (Figure 7B).

Reaction of MtBKatG and BpKatG with Peroxoaetic Acid and H$_2$O$_2$. To compare the properties of SynKatG with those of other KatGs, MtBKatG and BpKatG were first treated with peroxyacetic acid, revealing spectral changes quite different from those observed in SynKatG, including a red-shifted Soret band (to 415 nm) and two new maxima around 549 and 590 nm (Figure 8A,B) that were stable for at least 10 s. Conditions: 50 mM phosphate buffer at pH 7.0 and 25 $^\circ$C. (B) Spectral changes observed upon mixing of 2 $\mu$M ferric B. pseudomallei KatG with 100 $\mu$M PAA. The dashed line is for the ferric enzyme; subsequent spectra were taken after 1.3 ms (gray line), 200 ms (black line), 10 s (thick black line). Conditions: 50 mM phosphate buffer at pH 7.0 and 25 $^\circ$C. (C) pH dependence of compound I formation in M. tuberculosis. The time traces for the reaction of 2 $\mu$M ferric M. tuberculosis KatG with 200 $\mu$M PAA observed at 408 nm at various pH values are shown. (D) pH dependence of compound I formation in B. pseudomallei. The time traces for the reaction of 2 $\mu$M ferric B. pseudomallei KatG with 200 $\mu$M PAA observed at 407 nm at different pH values are shown.

Figure 7: Reaction of hydrogen peroxide with Synechocystis KatG compound I preformed with peroxyacetic acid. (A) Spectral changes observed in the reaction of 3 $\mu$M WT KatG compound I with 6 mM hydrogen peroxide. Spectra were taken immediately after mixing (1.3 ms), after 15 ms, and after 50 ms. Conditions: 50 mM phosphate buffer at pH 7.0 and 25 $^\circ$C. (B) Spectral changes upon mixing 3 $\mu$M WT KatG compound I with 10 mM hydrogen peroxide. The first spectrum is the compound I spectrum, and subsequent spectra were taken after 1.3 ms and after 20 ms. Conditions: 50 mM phosphate buffer at pH 6.0 and 25 $^\circ$C. (C) Time trace at 420 nm and single-exponential fit for the reaction in panel A. The inset shows the plot of the pseudo-first-order rate constant vs the concentration of hydrogen peroxide.

Figure 8: Reaction of M. tuberculosis and B. pseudomallei KatG with PAA. (A) Spectral changes observed upon mixing of 2 $\mu$M ferric M. tuberculosis KatG with 200 $\mu$M PAA. The thick gray line is for the ferric enzyme; subsequent spectra were taken after 1.3 ms (gray line), 220 ms (black line), and 1.1 s (thick black line). Conditions: 50 mM phosphate buffer at pH 7.0 and 25 $^\circ$C. (B) Spectral changes observed upon mixing of 2 $\mu$M ferric B. pseudomallei KatG with 100 $\mu$M PAA. The dashed line is for the ferric enzyme; subsequent spectra were taken after 1.3 ms (gray line), 200 ms (black line), 1.9 s (thick black line), and 10 s (thick gray line). Conditions: 50 mM phosphate buffer at pH 7.0 and 25 $^\circ$C. (C) pH dependence of compound I formation in M. tuberculosis. The time traces for the reaction of 2 $\mu$M ferric M. tuberculosis KatG with 200 $\mu$M PAA observed at 408 nm at various pH values are shown. (D) pH dependence of compound I formation in B. pseudomallei. The time traces for the reaction of 2 $\mu$M ferric B. pseudomallei KatG with 200 $\mu$M PAA observed at 407 nm at different pH values are shown.
In contrast to SynKatG, the reactions of both MtbKatG and BpKatG with PAA were pH-dependent and the kinetics were not monophasic at all pH values. At both acidic and alkaline pHs, the reaction of MtbKatG with PAA was biphasic and slower than at pH 7 (Figure 8C). The kinetics of oxidation of BpKatG by PAA were slower than for MtbKatG, revealing greater complexity, including an initial hypochromicity of the Soret band at 407 nm followed by an increase in absorbance and red shift to 415 nm (Figure 8D). The time trace of the changes at 407 nm in combination with the other spectral transitions suggests the existence of a transient intermediate in the reaction pathway leading to the compound I species with the observed spectral features.

Mixing of MtbKatG and BpKatG with H$_2$O$_2$ at pH 8.5 produced intermediates with the same spectral features that were observed for SynKatG at pH 8.5 and 7.0 (418 and 520 nm) for as long as H$_2$O$_2$ was being degraded (data not shown). At pH 7.0, mixing of both MtbKatG and BpKatG with H$_2$O$_2$ gave rise to spectra very similar to that of SynKatG at pH 5.6 (Figure 9A). For example, the reaction of 2 μM MtbKatG with 10 mM hydrogen peroxide at pH 7.0 caused, within 1.3 ms and lasting for 600 ms, a red shift of the Soret band (416 nm), the appearance of two peaks around 545 and 580 nm, and a diminished CT1 band. Ultimately, the absorbance increased generally without a change in the position of the maxima at 416, 545, and 580 nm, and even after depletion of 10 mM H$_2$O$_2$ (1.8 s; see the inset of Figure 9A), these spectral features persisted (Figure 9A, spectra taken after 2.5 s). The reaction of ferric MtbKatG with H$_2$O$_2$ at pH 5.6 led to spectral changes similar to those observed at pH 7.0 (Figure 9B), except that the general increase in absorbance occurred faster (within 100 ms). After complete dismutation of H$_2$O$_2$, the spectrum remained almost identical to that resulting from PAA-mediated oxidation of MtbKatG (415, 549, and 590 nm). Similar results were obtained with BpKatG.

Compared to that of SynKatG, the degradation of hydrogen peroxide by both MtbKatG and BpKatG was slower at pH 5.6, taking 10 s to dismutate 10 mM H$_2$O$_2$ compared to 2 s for SynKatG. The reaction of BpKatG with H$_2$O$_2$ was also tested at pH 4.5, made possible by its greater resistance to acidic conditions compared to SynKatG. A pH of 4.5 is the pH optimum for the peroxidase activity in BpKatG, and catalase activity is decreased to 30–40% of maximum levels at pH 6.5 but still higher than at pH 8.5 (37). The resulting spectral features of the intermediate were similar to those observed at pH 5.6 and 7.0 (415, 548, and 580 nm), but the reaction was slower with the spectrum at 1.3 ms, suggesting a mixture of the ferric enzyme and the intermediate. The reaction followed pseudo-first-order kinetics, and the bimolecular rate constant determined at a single concentration of hydrogen peroxide (1 mM) was 1.3 × 10$^4$ M$^{-1}$ s$^{-1}$.

The final step was to investigate the reaction of compound I of BpKatG, generated using PAA, with H$_2$O$_2$. No spectral changes were observed using a moderate excess of H$_2$O$_2$, but a large excess (1000-fold range) resulted in the formation of intermediates with the same spectral features and pH dependence (Figure 10) as those formed in the direct mixing of the ferric protein with hydrogen peroxide (Figure 9). The transition between the spectral features of PAA-generated compound I of BpKatG and the spectral features of the intermediate dominating in the presence of H$_2$O$_2$ could be fitted to a single-exponential equation which yielded a second-order rate constant of 1.7 × 10$^4$ M$^{-1}$ s$^{-1}$ at both pH 6.5 and 8.5.

**DISCUSSION**

Despite catalyzing the same reaction (2H$_2$O$_2$ → 2H$_2$O + O$_2$), heme-containing monofunctional catalases and bifunctional catalase-peroxidases do not share sequence or structural
similarities, raising the question of whether the reaction pathways are similar or different. Whereas catalases have been the subject of study for decades, the interest in catalase-peroxidases has developed more recently, in part because of its role in mediating isoniazid resistance in *M. tuberculosis*, but also from the standpoint of determining how an enzyme which so closely resembles a class I peroxidase can dismutate H₂O₂ at reasonable rates. The determination of crystal structures of KatGs, now from four different organisms, has led to the identification of several catalase-specific residues, subsequently confirmed by site-directed mutagenesis studies. Such unique features have suggested a number of unusual mechanisms controlling the catalase pathway, but a clear picture of the reaction pathway has remained elusive. To address this question, a comprehensive kinetic and spectral investigation of the reaction of three different catalase-peroxidases and one monofunctional catalase with H₂O₂ and PAA using stopped-flow techniques has been carried out and correlated with the kinetics of H₂O₂ degradation also monitored by stopped-flow spectroscopy.

The rate of H₂O₂ degradation by BLC is much faster than the rate exhibited by KatGs, but in both cases, the kinetics can be explained by a simple reaction pathway involving reactions 3 and 4. Changing the H₂O₂ concentration in the assay from below to higher than the apparent *Kₘ* of KatGs caused a change in the kinetic pattern of H₂O₂ degradation consistent with the change from a substrate-unsaturated to substrate-saturated state. Unfortunately, it was technically not possible to raise the H₂O₂ concentration above the apparent *Kₘ* for monofunctional catalases in the stopped-flow system to determine if they responded similarly. However, the overall reaction pathway involving H₂O₂ binding provides a reasonable explanation for the kinetic responses of the two enzymes. On the other hand, the very different pH profiles of the two classes of enzymes suggest some fundamental differences.

These differences are also evident in the spectral features of reaction intermediates formed during H₂O₂ dismutation by the two classes of enzymes which differ significantly. Two oxidized products have been observed after reaction of BLC with peroxoacetic acid, an oxoferryl prophyrin radical species and a presumed hydroxoferryl protein radical species (1, 16). Neither of these species is evident in the reaction with H₂O₂ because the rate of reaction 2 is so much faster than the rate of reaction 1 that compound I does not accumulate. However, compound I preformed with peroxoacetic acid is reduced to the ferric state by H₂O₂ at a rapid rate, confirming that it is an intermediate in the catalatic pathway.

The much slower turnover rate for the catalatic reaction in KatGs compared to monofunctional catalases suggested that it might be possible to capture and characterize reaction intermediates of KatG with H₂O₂. This proved to be the case with the rapid appearance, within 1.3 ms of mixing H₂O₂ with enzyme, of the spectral signatures of two low-spin species that are different from the spectra of the compound I species of both BLC and KatG generated by peroxoacetic acid. Specifically, at pH 8.5, the spectra exhibited maxima at 418 and 520 nm, while at pH 5.6, the spectra exhibited maxima at 415, 545, and 580 nm; both spectra had lost the CT1 band at 640 nm. The relative proportions of the two intermediates varied at intermediate pHs, depending on the pH and the KatG. Maximal conversion of the enzyme into the reaction intermediate required saturation of the enzyme with substrate ([H₂O₂] > apparent *Kₘ*).

The spectrum with features at 418 and 520 nm does not resemble the spectrum of any reaction intermediate previously reported in peroxidases or catalases. On the other hand, the spectrum with features at 415, 545, and 580 nm of the intermediate predominating at acidic pH resembles the spectra of compound III of plant peroxidases (32), the ferrous form of WT and the Y249F variant of SynKatG treated with O₂ (27), the ferric form of MtbKatG treated with superoxide (33), compound II of MtbKatG treated with excess H₂O₂ (13), the Y238F variant of BpKatG treated with peroxoacetic acid (unpublished data), several KatG variants treated with a small excess of H₂O₂ (8, 9, 13), HRP (28) [but not MtKatG (34)] at alkaline pH with a hydroxyl ion distal ligand, *Arthromyces ramosus* peroxidase with NH₂OH bound (35), and finally, to some extent, the inactive N-terminal domain of KatG of *E. coli* (416, 536, and 568 nm) (36). In the latter case, incubation of the N-terminal domain with a separately expressed C-terminal domain resulted in a partial restoration of both catalase and peroxidase activities as well as high-spin spectral features of wild-type KatG (6). In the case of the NH₂OH complex with the peroxidase, the crystal structure of the complex suggests coordination of the nitrogen atom to the heme iron and hydrogen bonding of the hydroxyl group with the distal histidine possibly representative of compound “0” or the H₂O₂—peroxidase complex prior to the reaction (35). The conclusion here should be that compound III-like spectra are not uncommon and may be exhibited by different six-coordinate low-spin structures.

The crystal structures of a number of catalase, peroxidase, and catalase-peroxidase peroxoacetic acid-generated reaction intermediates have recently been reported that reveal Fe—O bond lengths longer than the value of 1.65 Å expected for a classical compound I (Por⁴⁺Fe⁴⁺—O) structure. *Micrococcus lysodeikticus* catalase (37), *Helicobacter pylori* catalase (2IQF, manuscript in review), CCP (38), and BpKatG (39) were converted to intermediates with Fe—O bond lengths of 1.82, 1.85, 1.87, and 1.93 Å, respectively. In all cases, the longer length was explained in terms of a PorFeIV–O structure. Unfortunately, the situation has been complicated somewhat by QM and QM/MM calculations that postulate the existence of six isomers of horseradish peroxidase compound II, of which the two experimentally observed reaction intermediates, Fe⁵⁺=O and Fe⁴⁺—OH, are the least stable while the singlet and triplet states of the Por⁴⁺Fe⁴⁺—OH and Por⁴⁺Fe⁴⁺—OH₂ complexes are more stable (41). EPR studies have revealed a number of radical-based reaction intermediates that are formed during the treatment of catalases, peroxidases, and catalase-peroxidases with peroxoacetic acid or H₂O₂. The classic compound I species has a radical on the porphyrin, a result of a one-electron transfer to the iron (PorFe⁴⁺=O → Por⁴⁺Fe⁴⁺=O). This has been observed in virtually all heme peroxidases and catalases. However, specific enzymes in all classes also support the migration of an electron from the protein into the heme, quenching the porphyrin radical and producing a protein radical based on either a Trp or Tyr residue (10, 16–19, 41).
32). In such cases, protonation of the oxoferryl to form a hydroxoferryl species occurs rapidly \((\text{PorFe}^{IV}=\text{O}+\text{H}^+ \rightarrow \text{PorFe}^{IV}=\text{OH})\). In SynKatG, W106 has been identified as the site of a Trp radical while the location of the Tyr remains unidentified. The diversity of radical sites even in the same class of enzymes is evident in the identification of the surface-situated Y353 as a radical site in MiKatG (42).

Specific tyrosines have been identified as radical sites in CCP and lignin peroxidase (32).

The proximity of the KatG-specific adduct (MYW) to the reaction center stacked just 3.4 Å above the heme has led to conjecture about its role in the reaction. One proposal is that it forms one component of a molecular switch inductively controlling the catalase reaction (39). Arg426 can adopt two conformations depending on the pH and the oxidation state of the heme (39). In the Y conformation favored at pH > 6.5, Arg429 is in ionic association with the adduct. Conformation Y is in equilibrium with conformation R, which is favored at pH < 6.5 and predominates in KatG oxidized by PAA. Formation of an adduct radical as an intermediate during MYW formation has been proposed (30), but no radical has so far been experimentally associated with the adduct in EPR studies. This suggests that if an adduct radical is formed during catalysis, it is transient or short-lived, and freeze-quench EPR techniques will be required for its identification. Furthermore, the fact that the adduct is required for the catalase but not the peroxidase reaction suggests that if a transient radical on the adduct (MYW\(^++\)) is formed, it has a role only in the second stage of the catalase reaction, i.e., \(\text{H}_2\text{O}_2\) oxidation, and not in the formation of compound I or the associated electron transfer pathway leading to protein radical formation (10, 13). Alternative radical sites close to the heme, such as the proximal tryptophan observed in CCP (43), might also be considered.

In all of this, Arg426 plays a key role. On the one hand, its association with the tyrosinate ion on the adduct is required for optimal catalytic rates (10, 39). The role of the putative adduct radical becomes complicated as a result, because removal of an electron from the adduct during radical formation would reduce the negative charge on the adduct, thereby weakening its association with Arg426. Therefore, quenching of the adduct radical to re-form the tyrosinate ion followed by its reassociation with Arg426 must either be a concerted part of or precede compound I reduction. Second, the change between the 415, 545, and 580 nm (low pH) and 418 and 520 nm (high pH) species with a midpoint around pH 6.5 correlates well with the 50:50 mixture of R and Y conformations of the Arg426 side chain at pH 6.5 (44). The influence of Arg426 on the predominant reaction intermediate could be a result of inductive stabilization of a particular intermediate or even the selection of alternate reaction pathways.

In light of the foregoing, a number of schemes can be envisioned that provide possible identities to the intermediates responsible for the previously unknown 418 and 520 nm and compound III-like 415, 545, and 580 nm intermediates. They range from the relatively simple compound I–substrate complex (Por\(^+\)Fe\(^{III}=\text{O}+\text{H}_2\text{O}_2\)) to alternative compound I species that are active in \(\text{H}_2\text{O}_2\) oxidation (reaction 4) and have the porphyril radical quenched by an electron from the adduct (MYW\(^++\)PorFe\(^{IV}=\text{OH}\)), an alternative amino acid near the heme (AA\(^+\)PorFe\(^{IV}=\text{OH}\)), or both (MYW\(^++\)PorFe\(^{III}=\text{OH}–\text{AA}^+\)). Alternatively, because KatG is a class I peroxidase, it might utilize the modified catalytic mechanism of monofunctional peroxidases that includes formation of compound III (PorFe\(^{III}=\text{O} \rightleftharpoons \text{PorFe}^{III}=\text{O}^+\)) (32). In peroxidases, the slow decay of compound III is responsible for the low rate of catalatic turnover, but in KatG, a nearby radical located on the adduct (or an alternative site) [MYW\(^++\)((AA)^+PorFe^{III}=\text{O} \rightleftharpoons \text{MYW}^+((AA)^+PorFe^{III}=\text{O}^+)\)] guarantees both a high turnover rate and the release of molecular oxygen. In any case, the pH-dependent change in the reaction intermediate can be explained by simple protonation and/or deprotonation of several intermediates or, alternatively, by different intermediates being stabilized at different pHs.

In summary, spectra suggestive of two different reaction intermediates, depending on pH, appear after KatGs are mixed with \(\text{H}_2\text{O}_2\). One of the intermediates has not been reported previously, and it is clearly different from the signatures of intermediates generated after peroxoacetic acid treatment. Alternative techniques, including possibly freeze-quench EPR and Mössbauer spectroscopy, will have to be applied to differentiate among the possibilities.

REFERENCES


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chapter three

Probing hydrogen peroxide oxidation kinetics of wild-type
*Synechocystis* catalase-peroxidase (KatG) and selected variants

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Probing hydrogen peroxide oxidation kinetics of wild-type *Synechocystis* catalase-peroxidase (KatG) and selected variants

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Running title: Hydrogen peroxide oxidation by catalase-peroxidase
Abbreviations: KatG, catalase-peroxidase; BLC, bovine liver catalase; PAA, peroxoacetic acid; RFQ-EPR, rapid freeze-quench electron paramagnetic resonance; HS, high-spin, LS, low-spin; 5-c, five coordinated; 6-c, six coordinated; $k_{on}$, apparent bimolecular rate constant of cyanide binding to ferric heme protein; $k_1$, apparent bimolecular rate constant of compound I formation; $k_2$, apparent bimolecular rate constant of the transition of compound I to ferric KatG.

Key words: Catalase-peroxidase; hydrogen peroxide reduction; hydrogen peroxide oxidation; catalase activity; compound I; cyanide complex; stopped-flow spectroscopy.
Abstract
Catalase-peroxidases (KatGs) are unique bifunctional heme peroxidases that exhibit peroxidase and substantial catalase activities. However, the reaction pathway of hydrogen peroxide dismutation, including the electronic structure of the redox intermediate that actually oxidizes \( \text{H}_2\text{O}_2 \), is not clearly defined. Several mutant proteins with diminished overall catalase but wild-type-like peroxidase activity have been described in the last years. However, understanding of decrease in overall catalatic activity needs discrimination between reduction and oxidation reactions of hydrogen peroxide. Here, by using sequential-mixing stopped-flow spectroscopy, we have investigated the kinetics of the transition of KatG compound I (produced by peroxoacetic acid) to its ferric state by trapping the latter as cyanide complex. Apparent bimolecular rate constants (pH 6.5, 20°C) for wild-type KatG and the variants Trp122Phe (lacks KatG-typical distal adduct), Asp152Ser (controls substrate access to the heme cavity) and Glu253Gln (channel entrance) are reported to be \( 1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \), \( 3.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \), \( 3.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \), and \( 8.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \), respectively. These findings are discussed with respect to steady-state kinetic data and proposed reaction mechanism(s) for KatG. Assets and drawbacks of the presented method are discussed.
**Introduction**

Typical (monofunctional) catalases (EC 1.11.1.6) and catalase-peroxidases (KatGs, EC 1.11.1.7) have neither sequence nor structural homology, but both catalyze the dismutation of hydrogen peroxide: $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$. In typical catalases the *catalatic* mechanism is well characterized with conventional compound I [oxoiron(IV) porphyrin π-cation radical intermediate] being responsible for hydrogen peroxide oxidation [1]. The reaction pathway in KatGs is not as clearly defined. Like in monofunctional catalases, reaction of KatGs with alkyl peroxides generates an oxoiron(IV) porphyrin π-cation radical species, which rapidly transforms to a protein radical species [2-6]. Recently, it has been demonstrated by stopped-flow spectroscopy in combination with rapid-freeze-quench electron paramagnetic resonance (RFQ-EPR) spectroscopy that the protein radical is located, at least transiently, at the KatG-typical covalent distal side adduct (Trp-Tyr-Met) [6, 7]. Reaction of this redox intermediate with hydrogen peroxide constitutes the second phase of the dismutation in which $\text{H}_2\text{O}_2$ acts as two-electron reductant and dioxygen is released. In detail, it has been proposed that the oxoferryl heme rapidly reacts with hydrogen peroxide to produce oxyferrous $[\text{Fe(II)}-\text{O}_2 \leftrightarrow ]$ heme that decomposes to ferric KatG and superoxide, the latter being oxidized to $\text{O}_2$ by the Trp-Tyr-Met adduct radical [5, 6]. In sum, this mechanism is non-scrambling (i.e. both oxygen atoms derive from the same molecule of hydrogen peroxide) as has been demonstrated recently [8].

In order to understand the impact of mutations on the *catalatic* activity of KatG it is necessary to distinguish between the role of $\text{H}_2\text{O}_2$ as oxidant and as reductant. The heterolytic cleavage of $\text{H}_2\text{O}_2$ (i.e. compound I formation) is common to all heme peroxidases and catalases and is known to be very fast (~$1-5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) [1, 9, 10], whereas efficient $\text{H}_2\text{O}_2$ oxidation is only catalyzed by catalases and KatGs [1]. Typically, for dismutating enzymes (counts also for superoxide dismutases) it is difficult to measure the kinetics of the two half reactions since adding of substrate to either redox intermediate immediately promotes the enzyme to cycle. Here, we have adapted the sequential stopped-flow instrument in order to measure the kinetics of the two-electron reduction of pre-formed compound I with hydrogen peroxide. In detail, wild-type KatG and selected variants with great variability of *catalatic* activity were oxidized by peroxyacetic acid, to compound I, which was immediately reduced by hydrogen peroxide in the presence of cyanide in order to trap formed ferric KatG as low-spin (LS) complex. This allowed for the first time extraction of apparent $\text{H}_2\text{O}_2$ oxidation rates by KatG compound I. The
corresponding bimolecular rates are presented and compared with published overall kinetic parameters of KatG. The effect of mutations on the H₂O₂ oxidation reaction is discussed with respect to proposed reaction mechanism(s).

**Materials and Methods**

*Reagents and recombinant protein production.* Standard chemicals and biochemicals were obtained from Sigma at the highest grade available. Hydrogen peroxide was obtained from Sigma and its concentration was determined using an extinction coefficient at 240 nm of 39.4 M⁻¹ cm⁻¹. Peroxoaetic acid (PAA) was a 39% solution supplied by Fluka. Its concentration was determined iodometrically. Bovine liver catalase was obtained from Sigma and used without further purification. Its concentration was determined using a molar extinction coefficient ε₄₀₄ nm of 105,000 M⁻¹ cm⁻¹ per heme [5]. Heterologous expression in *E. coli*, purification and characterization of recombinant wild-type KatG and variants was reported recently: wild-type [11], Trp122Phe [12], Asp152Ser [13] and Glu253Gln [14], respectively.

*Stopped-flow spectroscopy.* Transient-state measurements were performed using a sequential-mixing SX.18MV stopped-flow spectrophotometer (Applied Photophysics Ltd.) equipped with a 1 cm observation cell thermostatted at 20°C and pH 6.5, the maximum of the catalatic activity. Calculation of pseudo-first-order rate constants (kₚₒₛₛ) from experimental time traces was performed with the SpectraKinetic work station (Version 4.38) interfaced to the instrument. The substrate or ligand concentrations were at least five times that of the enzyme to allow determination of pseudo first-order rate constants. Second-order rate constants were calculated from the slope of the linear plot of the pseudo first-order rate constants versus substrate concentration. To follow spectral transitions, a PD.1 photodiode array accessory (Applied Photophysics) connected to the stopped-flow machine together with Xscan diode array scanning software (Version 1.07) were utilized.

The conventional stopped-flow technique was used to measure the kinetics of cyanide binding to the ferric heme proteins. Final concentrations: 1 µM KatG and 10–200 µM cyanide in 50 mM phosphate buffer, pH 6.5. The first data point was recorded 1.5 ms after mixing, and 2000 data points were accumulated. The calculated kᵦₒₛₜ values (followed at a wavelength with largest absorption difference between high-spin and low-spin species) were fitted to the expression kᵦₒₛₜ = kₒₙ[CN⁻] + kₒᵦᵣᵦ, where kₒₙ is the rate of cyanide binding.
and $k_{\text{off}}$ the rate of dissociation of cyanide from the Fe(III) species. Values for the equilibrium dissociation constants were calculated using $K_D = k_{\text{off}} / k_{\text{on}}$.

The sequential-mixing stopped-flow technique was used to measure compound I reduction by hydrogen peroxide in the presence of sodium cyanide. In the first step native KatG and peroxoacetic acid (PAA) were premixed in the aging loop and after a defined delay time (determined by maximum hypochromicity at the Soret maximum) $\text{H}_2\text{O}_2$ and cyanide were added. In the beginning, 6 µM enzyme were mixed with an excess of PAA (20 to 66-fold excess) to elucidate maximum hypochromicity and optimum delay time. Finally, pre-formed compound I was mixed with various concentrations of hydrogen peroxide ranging from 10 µM to 200 µM (Trp122Phe: 20 µM to 10 mM) in the presence of 1 mM cyanide (50 mM phosphate buffer, pH 6.5).

**Results**

**Kinetics of cyanide binding to ferric KatG.** Since cyanide was intended to be used for trapping ferric KatG formed by compound I reduction, it was necessary to re-evaluate the kinetics of cyanide complex formation of native wild-type and mutant KatG. Ferric 5-coordinated (5-c) high-spin (HS) KatG from *Synechocystis* has its Soret band at 406 nm, Q-bands at 502 and 545 nm and a CT1 band (i.e. a porphyrin-to-metal charge transfer band) at 635 nm [10]. Upon cyanide binding the Soret peak shifted to 422 nm (isosbestic point at 414 nm) and a new prominent peak around 540 nm was formed. Formation of the low-spin (LS) complex was followed at 427 nm (corresponding to the largest difference between the high-spin and the low-spin species) and was monophasic. The apparent bimolecular rate constant, $k_{\text{on}}$, was calculated to be $(5.1 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.5 and 20°C. With Glu253Gln both the spectral characteristics of the high- to low-spin transition as well as the kinetics of complex formation were similar to wild-type KatG (Table 1).

Substitution of Trp122 by Phe not only inhibits formation of the KatG-typical covalent adduct [15] but also decreases formation of 6-c HS and LS species [16]. Compared to wild-type KatG binding of cyanide (Soret band of LS complex at 419 nm) was significantly slower, namely $(3.9 \pm 0.4) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. In contrast to the other proteins investigated here, cyanide binding to Asp152Ser was biphasic with a rapid first phase responsible for at least 85% of absorbance increase. This confirms already published data [13]. By fitting the first rapid phase with a single-exponential equation pseudo first-order rate constants, $k_{\text{obs}}$, were obtained that linearly increased with the concentration of cyanide.
The calculated apparent bimolecular rate constant was \((1.9 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\), about five times faster than that of wild-type KatG.

Table 1. Kinetic constants for compound I formation \((k_1)\) and reduction \((k_2)\) as well as cyanide complex formation of recombinant wild-type KatG from *Synechocystis* PCC 6803, selected variants (Glu253Gln, Asp152Ser, Trp122Phe) and bovine liver catalase, BLC (pH 6.5, 20°C). n.d., not determinable.

<table>
<thead>
<tr>
<th>Compound I formation</th>
<th>Wild-type</th>
<th>Glu253Gln</th>
<th>Asp152Ser</th>
<th>Trp122Phe</th>
<th>BLC</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cyanide binding</th>
<th>(k_{on})</th>
<th>(k_{off}) (s(^{-1}))</th>
<th>(K_D) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>51</td>
<td>7.6</td>
<td>15</td>
</tr>
<tr>
<td>Peroxoadic acid</td>
<td>82 [14]</td>
<td>10.7</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>7.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>3.9</td>
<td>2.9</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>3.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound I* reduction</th>
<th>Hydrogen peroxide (in presence of cyanide)</th>
<th>(k_{cat}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>1.2</td>
<td>3500 [12]</td>
</tr>
<tr>
<td>Peroxoadic acid</td>
<td>0.86</td>
<td>890 [14]</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>200 [13]</td>
</tr>
<tr>
<td></td>
<td>0.003</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>1100</td>
<td>212 000 [20]</td>
</tr>
</tbody>
</table>

* Formed with peracetic acid

In addition to the \(k_{on}\) values Table 1 depicts also \(k_{off}\) and \(K_D\) values for the corresponding cyanide complexes. Cyanide complexes of KatG are relatively stable with dissociation constants in the micromolar region. For comparative purposes we have also investigated cyanide binding to ferric bovine liver catalase (shift of Soret band from 404 nm to 420 nm) and calculated \(k_{on}\) to be \((1.1 \pm 0.03) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) (Table 1).

*Kinetics of transition of compound I to ferric KatG.* In the following sequential stopped-flow experiments cyanide was present in order to suppress cycling of catalase-peroxidase with excess \(\text{H}_2\text{O}_2\). Principally, ferric KatG (formed by two-electron reduction of compound I) has two possibilities to react, namely either with \(\text{H}_2\text{O}_2\) to compound I or with cyanide to the corresponding LS complex at rates summarized in Table 1. Assuming that the (unknown) rate of compound I formation by \(\text{H}_2\text{O}_2\) in KatG is of the same order of magnitude as in other heme peroxidases \((k_1 \sim 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\) [10]), the cyanide...
concentration had to be at least 50-times higher than that of added H₂O₂. Typically, with wild-type KatG concentrations of H₂O₂ <100 µM and 1 mM NaCN were used.

Figure 1A shows the spectral transition of 3 µM compound I mixed with 100 µM hydrogen peroxide in the presence of 1 mM cyanide. Oxidation of ferric KatG with peroxoacetic acid to compound I has been shown to give rise to a calculated reaction rate of 2-7 × 10⁴ M⁻¹ s⁻¹ depending on the source of KatG [2, 12, 17, 18]. The spectral signatures of the resulting Synechocystis intermediate included a 40-50 % hypochromicity of the Soret band and two distinct bands at around 604 and 643 nm, which were attributed to an oxoiron(IV) porphyrin cation radical species [5, 11]. Upon its mixing with hydrogen peroxide in the presence of cyanide the Soret band shifted directly to 420 nm (apparent isosbestic points at 403 and 464 nm) and a new peak at 540 nm appeared, closely resembling the spectrum of the cyanide complex (Figure 1A).

The reaction was monophasic and could be fitted using a single-exponential equation. A typical time trace is shown in Figure 1B. Plotting these pseudo-first-order rate constants versus H₂O₂ concentration (Figure 1C) yielded a linear correlation that allowed calculation of the apparent second-order rate, $k_2$, from the slope: $(1.2 \pm 0.2) \times 10^4$ M⁻¹ s⁻¹ at pH 6.5 and 20°C. This clearly suggests that the rate limiting step in the consecutive Reactions 1 & 2 is compound I reduction by H₂O₂ (i.e. release of dioxygen). Figure 1D depicts the calculated concentration changes of the redox intermediates involved in this reaction sequence. Since $k_{on} > k_2$, ferric KatG did not accumulate but was rapidly transferred to its LS complex.

\[
\text{compound I} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{H}_2\text{O} + \text{O}_2 \quad \text{Reaction 1}
\]
\[
\text{Fe(III)} + \text{CN}^- \rightarrow \text{Fe(III)}-\text{CN}^- \quad \text{Reaction 2}
\]

Decrease in the concentration of compound I is accompanied by an increase of the KatG-cyanide complex (Figure 1D), whereas the concentration of ferric KatG is highest at around 6 nM (<< 1% of total KatG protein) in the first phase of reaction (Figure 1E). This has been calculated based on the fact that (i) $k_{on} > k_2$, and (ii) ferric KatG reaches a steady-state level:

\[
k_2\text{[compound I][H}_2\text{O}_2] = k_{on}\text{[Fe(III)][CN}^-]
\]
\[
d\text{[Fe(III)]}/dt = 0 = k_2\text{[compound I][H}_2\text{O}_2] - k_{on}\text{[Fe(III)][CN}^-]
\]
\[
\text{[Fe(III)}]_{\text{steady-state}} = \{k_2\text{[compound I][H}_2\text{O}_2]}/\{k_{on}\text{[CN}^-]\}
\]
Under assumption that in the initial phase of reaction the concentrations of compound I, H$_2$O$_2$ (100 µM) and CN$^-$ (1 mM) are constant and using \( k_2 = (1.2 \pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_{\text{on}} = 5.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \), [Fe(III)]$_{\text{steady-state}}$ was calculated to be \(~6 \text{ nM} \) (out of 1.5 µM KatG). Thus, under these conditions ferric KatG does not contribute to the observed spectral transitions. As a consequence the compound I spectrum seemed to be transformed directly into that of the cyanide complex and the corresponding time traces could be fitted with a single-exponential equation. These calculations support our experimental setup for determination of H$_2$O$_2$ oxidation rates in catalase-peroxidases.

Similar spectral transitions were observed for the variant Glu253Gln and \( k_2 \) was calculated to be \((8.6 \pm 0.1) \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \) at pH 6.5 and 20°C. This corresponds to \(~72\% \) of the wild-type rate. In case of Trp122Phe high concentrations of H$_2$O$_2$ had to be added to compound I in order to induce a spectral shift of the Soret maximum from 409 nm to 417 nm and formation of peak at 540 nm (data not shown). The rate of transition was extremely slow \((30 \text{ M}^{-1} \text{ s}^{-1}, <0.3\% \) of wild-type rate\) thereby confirming previous data that clearly showed the importance of Trp122 (and of the intact KatG-typical Trp122-Tyr249-Met275 adduct) in catalatic activity, i.e. oxidation of H$_2$O$_2$ [10].
Figure 1  Vlasits et al.
Figure 1. Reaction between compound I of wild-type *Synechocystis* KatG with hydrogen peroxide in the presence of cyanide. (A) Spectral changes upon mixing 3 µM compound I (pre-formed with 200 µM PAA & delay time of 5 s) with 100 µM H₂O₂ and 1 mM NaCN in 50 mM phosphate buffer, pH 6.5 and 20°C. Spectra were recorded 1.3 ms, 100 ms, 280 ms, 430 ms, 880 ms, and 2.2 s after mixing. (B) Typical time trace at 427 nm and fitted using a single-exponential equation. Conditions: 1.5 µM KatG, 100 µM H₂O₂, 1 mM NaCN. (C) Plot of the pseudo-first order rate constants versus the concentration of hydrogen peroxide. (D) & (E) Calculated changes in concentration of redox intermediates involved the consecutive reaction compound I → ferric KatG → cyanide complex. Conditions as in (B).

In case of Asp152Ser, formation of the LS complex by addition of hydrogen peroxide and cyanide to compound I was biphasic (Supplemental Figure). Supplemental Figure B shows the time trace at 427 nm fitted with a single- (*thin line*) and a double-exponential (*thick line*) equation. In the latter case the second term was independent of the concentration of H₂O₂. With both fits very similar \( k_2 \) values could be obtained: \((3.4 \pm 0.1) \times 10^3\) M\(^{-1}\) s\(^{-1}\) (~28% of wild-type rate).

Additionally, we have probed the reaction of BLC compound I pre-formed with PAA and hydrogen peroxide in the presence of cyanide. Within milliseconds the BLC cyanide complex was formed with typical peaks at 420 nm, 550 and 585 nm, respectively (Figure 2A). Similar to KatG the rate of complex formation strongly depended on the H₂O₂ concentration and the time traces could be fitted to a single-exponential equation (Figure 2B). This allowed to calculate apparent \( k_2 \) to be \(1.1 \times 10^7\) M\(^{-1}\) s\(^{-1}\) (Figure 2C) which compares with a published rate calculated from steady-state data \((3.2 \times 10^7\) M\(^{-1}\) s\(^{-1}\) [19]). Despite the fact that in case of BLC \( k_2 > k_{on} \), compound I reduction was the rate-limiting step under the actual conditions. Since in BLC \( k_2 \) is more than two orders of magnitude faster than in KatG, the used H₂O₂ concentrations (< 15 µM) were low (at least 70 times lower than cyanide concentration of 1 mM). The experimental approach is supported by calculations of the concentration of the redox intermediates in the time course of reaction (Figures 2D & E). Ferric BLC did not accumulate but was effectively trapped in its LS cyanide complex. Its highest concentration in the initial phase of reaction was calculated to be ~0.03 µM (out of 1.5 µM KatG protein) (Figures 2D & E).
Figure 2  Vlasits et al.
Figure 2. Reaction between compound I of bovine liver catalase (BLC) with hydrogen peroxide in the presence of cyanide. (A) Spectral changes upon mixing 1.5 µM BLC compound I (pre-formed with 100 µM PAA & delay time of 4 s) with 2 µM H₂O₂ and 1 mM NaCN in 50 mM phosphate buffer, pH 6.5 and 20°C. Spectra were recorded immediately after mixing (1.3 ms), 22 ms, 50 ms, 73 ms, 110 ms, and after 4 s. (B) Typical time trace at 425 nm including fit using a single-exponential equation. Conditions: 1.5 µM KatG, 2 µM H₂O₂, 1 mM NaCN. (C) Plot of the pseudo-first order rate constants versus the concentration of hydrogen peroxide (2-15 µM). (D) & (E) Calculated changes in concentration of redox intermediates involved the consecutive reaction compound I $\rightarrow$ ferric BLC $\rightarrow$ cyanide complex. Conditions as in (A).

Discussion

It has been demonstrated that mixing of compound I (prepared with PAA) of wild-type *Synechocystis* KatG with moderate levels of hydrogen peroxide (<100 µM) did not induce spectral transitions simply because (i) the enzyme immediately entered the catalatic cycle and (ii) the rate of compound I formation ($k_1$) was apparently higher than that of compound I reduction ($k_2$). However, mixing millimolar concentrations of H₂O₂ with pre-formed compound I or directly with ferric KatG in the stopped-flow instrument led to the formation of discrete LS species (absorbance maxima at 415 nm, 545 nm and 580 nm at low pH and 418 nm and 520 nm at high pH) that represented the dominating redox species during catalase turnover [5]. These kinetic and spectral investigations clearly demonstrated differences in the mechanism of hydrogen peroxide dismutation mediated by catalase-peroxidases and monofunctional catalases and might concern mainly the oxidation reaction of H₂O₂. However, the so far performed experiments did not allow to measure this individual reaction since addition of hydrogen peroxide immediately prompted both heme enzymes to cycle.

Here, based on the fact that native KatG and BLC form relatively stable cyanide complexes with $K_D$ values in the low micromolar region and that the rates of complex formation can easily be followed by conventional stopped-flow spectroscopy, we have decided to follow compound I reduction mediated by H₂O₂ in the presence of excess cyanide. This guaranteed that (i) ferric KatG did not accumulate and was immediately trapped in its LS complex, and (ii) the kinetics of cyanide complex formation was fully dependent on peroxide concentration. The findings demonstrate that the kinetics of transition of compound I to ferric KatG is about 2-3 orders of magnitude slower than the corresponding reaction in monofunctional catalases, reflecting the differences in the published $k_{cat}$ values [20]. Thus, monofunctional catalases are able to catalyze the two-electron oxidation of H₂O₂ more efficiently than KatGs. Another discrepancy between the two catalatically active enzymes concerns the relative rates of $k_1$ and $k_2$. In KatG $k_2 = 1.2 \times 10^4$ M⁻¹ s⁻¹ $<< k_1$, whereas in BLC $k_2 > k_1$. 

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Nevertheless, KatGs are the only heme enzymes with a peroxidase-typical active site architecture that can dismutate hydrogen peroxide at reasonable rates. In close correlation with the ability to oxidize H$_2$O$_2$ is the KatG-typical distal covalent adduct Trp122-Tyr249-Met275. Its disruption, that occurs upon exchange of Trp122 or Tyr249, converts a bifunctional KatG into a monofunctional peroxidase [12, 21]. In Trp122Phe very high amounts of hydrogen peroxide were necessary in order to reduce compound I and, finally, induce formation of the cyanide complex. The slow rate (30 M$^{-1}$ s$^{-1}$) underlines the importance of the adduct in the catalase turnover of KatG that has been proposed [5] and recently demonstrated by rapid freeze-quench electron paramagnetic resonance experiments [6]. Shortly, in KatG classical compound I [oxoiron(IV) porphyrin cation radical] is rapidly converted to an isoelectronic species by formation of the adduct radical [i.e. oxoiron(IV) adduct radical]. The latter rapidly reacts with the second H$_2$O$_2$ molecule to produce oxyferrous heme [Fe(II)-O$_2$ ↔ Fe(III)-O$_2^*$], which decomposes to ferric enzyme and superoxide, the latter being quantitatively oxidized at the adduct radical closing the adduct shell and releasing dioxygen.

The chosen experimental setup does not distinguish between the individual reaction steps during transition of compound I to ferric KatG and release of O$_2$. But based on the observation that (i) formation of the oxyferrous form is faster than release of dioxygen [6] and (ii) the fact that its spectral signatures could be trapped in stopped-flow experiments upon addition of high amounts of H$_2$O$_2$ to either ferric KatG or pre-formed compound I [5], it is reasonable to assume that the calculated rates in this work reflect the kinetics of the transition of oxyferrous KatG to the ferric form. In conventional (monofunctional) peroxidases the decay of the oxyferrous (i.e. compound III) form is very slow. In KatG the unique function of the adduct radical is to promote the oxidation of superoxide radical to 3O$_2$.

Another structural feature that enables KatG to be catalytically active is the architecture of the substrate channel. In contrast to typical peroxidases KatGs have a more deeply buried active site, and the proposed access route for hydrogen peroxide is provided by a channel that is similar but longer and more restricted than that in other heme peroxidases, very similar to monofunctional catalases [1, 14, 20]. Aspartate 152 is fully conserved in KatG and is situated in the heme distal cavity at the main access channel entrance. It has been suggested that it might modulate the H$_2$O$_2$ entry into the active site and its substitution by serine has significantly reduced the overall catalatic activity. This is
confirmed by the present study that showed a diminished (28% of wild-type) rate of transition of compound I of Asp152Ser to its native state.

The last variant chosen for this study was Glu253Gln because – despite its location far away from the active site at the entrance of the access channel – it has been demonstrated that it also exhibits decreased overall dismutation activity compared to the wild-type protein [14]. Interestingly, this was also reflected by the present study. Exchange of Glu253 had an effect on the rate of reduction of compound I to ferric Glu253Gln most probably by partially impaired substrate (electron donor) delivery. The acidic residue is conserved in KatGs and helps in maintenance of a (rigid) H-bonding network. The latter is a precondition for catalysis of H_{2}O_{2} dismutation [14].

Table 1 and Figure 3 demonstrate that both the apparent rate constants for compound I reduction obtained in this transient kinetics study as well as the turnover numbers (k_{cat}) calculated from steady-state experiments follow the same order: wild-type KatG > Glu253Gln > Asp152Ser >> Trp122Phe. The discrepancies in absolute percentage values related to wild-type KatG (100%), i.e. the fact that the impact of the mutations on the overall turnover numbers is always more pronounced than on k_{2} values, suggest that effects of mutation(s) on substrate delivery might concern also compound I formation (k_{1}). In any case, the suggested method for the first time allows direct monitoring of the second half of the dismutation reaction of a catalase and is a valuable tool to study structure-function relationships in these unique bifunctional enzymes but also in monofunctional catalases.
Figure 3. (A) Reaction sequence that provides the basis for the experimental setup and (B) schematic comparison of catalytic turnover numbers and apparent bimolecular oxidation rates of H$_2$O$_2$ of wild-type KatG (100%: $k_{cat} = 3500$ s$^{-1}$ and $k_2 = 1.2 \times 10^4$ M$^{-1}$ s$^{-1}$) from *Synechocystis* and selected variants.
Acknowledgements
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References


**Supplemental Figure.** Reaction of *Synechocystis* KatG variant Asp152Ser compound I (prepared using peracetic acid) with hydrogen peroxide and cyanide. (A) Spectral changes upon mixing 1.5 µM compound I with 10 mM H$_2$O$_2$ in 1 mM NaCN. Spectra were recorded immediately after mixing (1.3 ms, bold black line), 32 ms (thin black line), 820 ms (dotted line), 3.1 s (thin grey line), and after 10 s (thick grey line). Conditions: 50 mM phosphate buffer at pH 6.5 and 20°C. (B) Time trace of the reaction in A, monitored at 427 nm and fitted using a single-exponential (thin line) and a double-exponential (bold line) equation. (C) Plot of the pseudo-first order rate constants, obtained from the double-exponential fit in B, versus the...
chapter four

Disrupting the H-bond network in the main access channel of catalase-peroxidase: effect on the redox thermodynamics of the Fe(III)-Fe(II) couple

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Disrupting the H-bond network in the main access channel of catalase-peroxidase: effect on the redox thermodynamics of the Fe(III)-Fe(II) couple

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**Abbreviations:** KatG, catalase-peroxidase; CcP, cytochrome c peroxidase; APX, ascorbate peroxidase; MPO, myeloperoxidase; HRP-C, horseradish peroxidase isoform C; ARP, *Athromyces ramosus* peroxidase; $E^{\circ}$, standard reduction potential (pH 7, 25°C); SHE, standard hydrogen electrode; $\Delta H^{\circ}_{rc}$, enthalpy change for the reaction center upon reduction of the oxidized protein; $\Delta S^{\circ}_{rc}$, entropy change for the reaction center upon reduction of the oxidized form; RR, resonance Raman; Fe-Im, iron-imidazole; ASA, accessible surface area.

**Keywords:** catalase-peroxidase; catalase activity; reduction potential; redox thermodynamics; enthalpy; entropy; heme cavity architecture; access channel; protein modeling.
Abstract

Catalase-peroxidases are the only heme peroxidases with substantial hydrogen peroxide dismutation activity. In order to understand the role of the redox chemistry in the bifunctional activity of these metalloproteins, catalytically active and inactive mutant proteins have been probed in spectroelectrochemical experiments. In detail, in a comparative study wild-type KatG from Synechocystis has been compared with variants with (i) disrupted KatG-typical adduct (Trp122-Tyr249-Met275), (ii) mutation of the catalytic distal His123-Arg119 pair, (iii) altered channel accessibility to the heme cavity (Asp152, Ser335) and modified charge at the channel entrance (Glu253). A valuable insight into the mechanism of reduction potential ($E^{\circ}$) modulation in KatG has been obtained from the factorization of the corresponding enthalpic and entropic components, determined from the analysis of the temperature dependence of $E^{\circ}$. Moreover, the model structures of the Fe(III) and Fe(II) forms of wild-type KatG from Synechocystis were computed using the available X-ray structures of KatGs from different origins as templates. Obtained results, discussed together for the published resonance Raman data on the strength of the proximal iron-imidazole bond as well as with catalytic properties of the corresponding mutants, clearly demonstrate that the absolute value of the midpoint potential of the Fe(III)/Fe(II) couple has no impact on catalase and peroxidase activity. Besides the role of the Trp-Tyr-Met adduct as radical site in catalysis, it is the architecture of the heme cavity and of the long and constricted substrate channel that distinguishes KatGs from monofunctional peroxidases. The role of maintenance of an ordered matrix of oriented water dipoles for H$_2$O$_2$ oxidation and the effect of its disruption is clearly seen by modification of enthalpic and entropic contributions to $E^{\circ}$ that reflect changes in polarity, electrostatics, continuity and accessibility of solvent to the metal center as well as solvent reorganization effects upon reduction.
**Introduction**

Catalase-peroxidases (KatGs) have raised considerable interest, since, despite having striking sequence homologies with heme peroxidases like ascorbate peroxidase (APX) or cytochrome c peroxidase (CcP) [1], they exhibit substantial catalase activity similar to monofunctional catalases. Thus, they are ideal model oxidoreductases to study structural modifications that enable a peroxidase to efficiently dismutate hydrogen peroxide. The available crystal structures of KatGs from *Haloarcula marismortui* (pdb code 1ITK), *Burkholderia pseudomallei* (pdb code 1MWV), *Mycobacterium tuberculosis* (pdb code 1SJ2), and *Synechococcus* PCC 7942 (pdb code 1UB2) [2-5] revealed that the principal organization of their active site is very similar to those of CcP [6] and APX [7, 8], including the proximal (His290-Trp341-Asp402; *Synechocystis* PCC 6803 numbering) and the distal (Arg119-Trp122-His123) triad (Figure 1). Unique to KatG and essential for catalatic activity (that is completely absent in both APX and CcP) is a highly conserved peculiar distal site adduct that includes Trp122-Tyr249-Met275 [2-5, 9] and plays a role as transient radical site in the catalatic turnover [10, 11]. In addition, at variance with typical peroxidases, but similar to monofunctional catalases, KatGs have a longer and more restricted access channel to the deeply buried heme b. KatG-specific large loop insertions build up this channel and also connect the distal and proximal domains. Large loop 1 (LL1) is of particular interest, since it contains a number of highly conserved residues that are essential for efficient H\textsubscript{2}O\textsubscript{2} oxidation. These include Tyr249 (part of the covalent adduct), Ile248 and Glu253, the latter creating an acidic entrance to the channel. Isoleucine 248 is hydrogen-bonded to KatG-specific Asp152 that, together with Ser335, forms the narrowest part of the channel and controls access to the distal heme cavity (Figure 1). Resonance Raman data and structural analysis demonstrated the existence of a very rigid and ordered structure built up by the interaction of these residues with distal and (via LL1) proximal amino acids, with the heme itself, and with the solute matrix in the channel. Disruption of these interactions typically affected the catalase but not the peroxidase activity of KatG [12, 13]. Here, we address the question to which extent the protein matrix and the ordered matrix of oriented water dipoles contribute to the reduction potential of the heme iron and in consequence to the catalatic activity of KatG. We have investigated the thermodynamics of the one-electron reduction, $E^{\circ}$, of the ferric heme in wild-type KatG from *Synechocystis* and in variants that lack (i) the KatG-typical adduct
(Trp122Phe, Tyr249Phe), (ii) the peroxidase-typical catalytic residues (Arg119Asn, His123Gln), as well as (iii) in variants involved in stabilization of the solute matrix of the access channel (Asp152Ser, Ser335Gly and Glu253Gln). Analysis of the temperature dependence of the corresponding reduction potentials, allowed factorization of the corresponding enthalpic and entropic components and in consequence of the protein- and solvent-based contributions to $E^{\circ}$. Moreover, we have computed the model structures of the Fe(III) and Fe(II) forms of wild-type KatG from *Synechocystis* using the available X-ray structures of KatGs as templates [2-5]. Obtained findings are compared with data from enzyme kinetics of the corresponding mutants as well as with those known from monofunctional peroxidases and, finally, discussed with respect to the unique H$_2$O$_2$ dismutation activity of KatG.

**Results**

The final model of catalase-peroxidase from *Synechocystis* PCC 6803 includes residues 49-754. Residues 1–48 are excluded since, in the multiple sequence alignment used (Supplemental Figure S1), they correspond to residues which are not resolved in the X-ray structures of the catalase-peroxidases used as templates. This N-terminal segment is presumably very flexible and is not conserved in the catalase-peroxidases family [14], as shown by the sequence alignment (see Supplemental Figure S1). Omitting this portion is unlikely to be relevant for the protein function.

As expected from both the high sequence identities (55%-72%) and sequence similarities (78%-88%) between target and templates, the fold of the modelled structure of *Synechocystis* catalase-peroxidase is very similar to that of the templates (rmsd range: 5.6 – 10.2 Å) and shows two structurally similar domains (Figure 1A), which are mainly formed by $\alpha$-helices: the N-terminal domain (residues 49–461) and the C-terminal domain (residues 462–754). Although having only 19% sequence identity (and about 30% sequence similarity), the two domains share a similar fold (the secondary structure elements of the N-terminal segment 88-439 superimpose to those in the C-terminal segment 475-746 with a 1.2 Å rmsd), which is common in the members of the bacterial and plant peroxidase families, including cytochrome $c$ peroxidase [6], ascorbate peroxidase [7, 8], and horseradish peroxidase C [15]. The topological arrangement of the $\alpha$-helices and $\beta$-sheets is largely conserved. In particular,
the conformations of both KatG-specific large loops LL1 and LL2 are fully conserved [2]. The main secondary structure elements found in the modelled structure are almost consistent with those predicted by the PredictProtein web service (see Supplemental Figure S1).

**Figure 1.** (A) Cartoon representation of the 3D modeled structure of KatG from *Synechocystis* PCC 6803. (B) Focus on the oxidized (left) and reduced (right) active sites of the *Synechocystis* PCC 6803 KatG models. The most relevant residues and the water molecules in the active site cavity are highlighted and labeled. Code: arrows: \(\beta\)-strands; barrels: \(\alpha\)-helices; red liquorice: heme group; bold liquorice: protein site residues; thin liquorice: water molecules; cyan: C atoms; blue: N atoms; red: O atoms; yellow: S atoms. This picture was drawn with the VMD software [73].

The single heme \(b\) group of the protein is buried inside the N-terminal domain and can be reached by the substrate through a narrow hydrophilic channel that prevents access of large substrates. In the C-terminal domain, the cleft corresponding to the heme site is occupied by the C-terminal loop 589-619. The extension of the active site cavity and the architecture of the heme site are similar to those of the templates and other class I peroxidases [2]. The conserved
key residues (R119, W122, and H123 in the distal pocket, H290, W341, and D402 in the proximal pocket, and S335) show an arrangement closely similar to that observed in the X-ray structures of catalase-peroxidases and class I peroxidases (Figure 1). The same holds for the distal covalent adduct formed by W122, Y249 and M275, which is peculiar to the catalase-peroxidase family [2]. The H-bond network and the arrangement of the water molecules within the active site cavity in the modelled structure of ferric Synechocystis KatG are consistent with those observed in the catalase-peroxidase templates (Figure 2).

The ferric and ferrous forms are highly similar: the overall rmsd is 0.10 Å, while the rmsd computed on their Cα chain is 0.04 Å. The computed rmsd values - taking into account only the heme and the conserved residues in the active site is 0.14 Å - clearly indicate that the most relevant structural differences between the models of the two redox states of the protein are found in the active site region (Figure 1B). These small local structural modifications do not significantly alter the solvent accessibility of the heme group, whose water accessible surface area (ASA) is similar in the reduced and in the oxidized protein (31Å² versus 30Å², respectively), as is its subdivision into hydrophobic and hydrophilic components (80% and 20% for the oxidized protein; 77% and 23% for the reduced protein). On the other hand, the water accessible surface area of the whole distal active site (including the heme and the residues R119, W122, H123, D152, Y249, which constrict the active site cavity) slightly increases upon Fe(III) reduction, passing from 77 Å² to 81 Å². Also the distribution of the hydrophilic and hydrophobic components is slightly altered (passing from 48% hydrophobic and 52% hydrophilic in the ferric protein to 43% hydrophobic and 57% hydrophilic in the ferrous enzyme), as a consequence of the small reduction-induced displacement of the side chains of residues H123, R119 and D152 (Figure 1B). Although the ASAs of the distal cavity in the two redox forms are comparable, the water molecule distribution in the distal cavity slightly changes upon Fe(III) reduction. In particular, the water molecules closest to the heme shift slightly away from the metal center (Figure 1B), in agreement with a decreased electrostatic interaction with the ferrous ion, thereby slightly increasing their H-bonding interactions with the aminoacids inside the distal cavity (Figure 2).
Figure 2, Vlasits et al.
Figure 2. (A) Detailed view of the active site of KatG from *Burkholderia pseudomallei* (*Synechocystis* numbers in parentheses) with the interacting water (W) molecules starting at the constriction of the channel. Water molecules are numbered 9 – 18 and correspond to the following structural water molecules of *Burkholderia*. W9, position 3086; W10, position 3180; W11, position 2653; W12, position 2511; W13, position 3138; W14, position 3071; W15, position 2764; W16, position 3897; W17, position 3085; W18, position 2229. (B) Detailed view of the main channel showing the residues investigated in this study, including the amino acids asparagine and proline, and the interacting water molecules. W9, W10 and W12 – W15 correspond to the positions in A. W1, position 3558; W2, position 2472; W3, position 2860; W4, position 3149; W5, position 3620; W6, position 4107; W7, position 3898; W8, position 3770. (C) Surface structure of the main channel of KatG. Green, heme; blue, Ser324 (Ser335); cyan, Asp141 (Asp152); yellow, Glu242 (Glu253); red, water molecules; W1 – W8 correspond to the positions in B and W9 – W13 to the positions in A. The figures were constructed using the coordinates deposited in the Protein Data Bank (accession code 1MWV).

The spectroscopic properties of ferric wild-type recombinant KatG from *Synechocystis* are indicative of predominant five-coordinate high-spin heme with the Soret band at 407 nm, Q-bands at 502 and 545 nm and a CT1 band (i.e. a porphyrin-to-metal charge transfer band) at 635 nm, very similar to those reported in the literature [13]. For comparison and in order to guarantee identical conditions, the standard reduction potential, $E^{\prime\prime}$, of the Fe(III)/Fe(II) couple of wild-type protein has been re-measured. Figure 3A depicts a representative family of spectra of ferric KatG at different applied potentials in the OTTLE cell. Ferric KatG is directly reduced to its ferrous form (spectral bands at 438 nm and 557 nm) with a clear isosbestic point at 420 nm. The calculated midpoint reduction potential for the Fe(III)/Fe(II) couple, determined from the corresponding Nernst plot (inset to Figure 3A), was identical to the value published recently (i.e. $-0.226 \pm 0.005$ V, 25°C and pH 7.0) [14]. Similarly, the ferric mutant proteins were exposed to different potentials and the one-electron reduction was followed spectroscopically. As a representative example, the spectral transition of the variant Asp152Ser that mimics CcP and exhibits significantly lower catalase activity compared to the wild-type protein [15] is shown in Figure 3B. Ferric Asp152Ser (Soret: 406 nm, CT1: 640 nm) is directly converted (isosbestic point at 417 nm) to its ferrous form (spectral bands at 438 nm and 557 nm). Similar to the wild-type protein and all other mutants investigated in this work, the slope of the linear Nernst plot was close to the theoretical value of $RT/F = 0.059$ V at 25°C and thus consistent with a one-electron redox reaction. The determined $E^{\prime\prime}$ of the Fe(III)/Fe(II) couple of Asp152Ser was $-0.208 \pm 0.010$ V (25°C and pH 7.0), 20 mV more positive than the wild-type protein. For details of spectral transitions, Nernst plots and calculation of $E^{\prime\prime}$ see Supplemental Figure S2. Table 1 summarizes the $E^{\prime\prime}$ values of the other variants.
Table 1. Thermodynamic parameters for Fe(III)$\rightarrow$Fe(II) reduction in wild-type *Synechocystis* KatG and several variants. For comparison purposes, the corresponding thermodynamic parameters of horseradish peroxidase isoform C (HRP-C), *Arthromyces ramosus* peroxidase (ARP), and myeloperoxidase (MPO) are presented. Average errors on $E^\circ$, $\Delta H^\circ$, and $\Delta S^\circ$ values are ±0.005 V, ±5 kJ mol$^{-1}$, and ±8 kJ mol$^{-1}$. In addition resonance Raman data in the low-frequency region as well as kinetic parameters of the catalase reaction are presented.

<table>
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<tr>
<th>Protein</th>
<th>$E^\circ$ (V)</th>
<th>$\Delta H^\circ$ (KJ mol$^{-1}$)</th>
<th>$\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta H^\circ/F$ (V)</th>
<th>$T\Delta S^\circ/F$ (V)</th>
<th>$\nu$(Fe-Im) (cm$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
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<td>n.d. [26]</td>
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<tr>
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<td>+166</td>
<td>-0.840</td>
<td>+0.513</td>
<td>202, 249 [23]</td>
<td>16 [26]</td>
<td>0.83 [26]</td>
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<td>+0.763</td>
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<td>60 [26]</td>
<td>17 [26]</td>
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<td>203, 251 [12]</td>
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<td>243 [39]</td>
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<td>+10</td>
<td>-0.031</td>
<td>+0.031</td>
<td>246 [42]</td>
<td>-</td>
<td>-</td>
</tr>
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*data from this work
Principally, the reduction potentials of Trp122Phe, Tyr249Phe, Ser335Gly and Glu253Gln were found to be more or less identical (-0.222 to -0.226 V) with that of the wild-type protein.

![Graph showing absorbance vs. wavelength (nm) for different conditions.](image)

**Figure 3.** (A) Electronic spectra of wild-type catalase-peroxidase from *Synechocystis* PCC 6803 obtained at various applied potentials at 25 °C. (B) Electronic spectra of the variant, KatG(D152S) measured at 25°C. The insets depict the corresponding Nernst plots, were X represents $[(A_{\lambda_{\text{ox}}} - A_{\lambda_{\text{red}}})/(A_{\lambda_{\text{ox}}} - A_{\lambda_{\text{red}}})]$ with $\lambda_{\text{ox}} = 406$ nm and $\lambda_{\text{red}} = 438$ nm for wild-type KatG, and $\lambda_{\text{ox}} = 406$ nm and $\lambda_{\text{red}} = 436$ nm for KatG(D152S).

By contrast, $E^\circ$ of both His123Gln (-0.319 ± 0.005 V) and Arg119Asn (-0.305 ± 0.005 V) exhibited a significantly more negative reduction potential than wild-type KatG.

To gain a deeper insight into the mechanism of $E^\circ$ modulation, the temperature dependence of the reduction potential was investigated (Figure 4). This allows factorization of the corresponding enthalpic ($\Delta H^\circ_{\text{rc}}$) and entropic ($\Delta S^\circ_{\text{rc}}$) components of the reduction reaction. In all investigated proteins, the oxidized state was enthalpically stabilized over the reduced state, although the relative contributions of $\Delta H^\circ_{\text{rc}}$ were different. Without consideration of His123Gln and Arg119Asn, the enthalpic contribution to the negative $E^\circ$ value was in the range of -41 mV to -176 mV following the hierarchy Ser335Gly < Trp122Phe < Asp152Ser < Glu253Gln < wild-type KatG (Table 1). In both His123Gln and Arg119Asn the enthalpic contribution to the negative $E^\circ$ value was very high (-840 mV and -1078 mV, respectively).

With the exception of His123Gln and Arg119Asn, reduction of wild-type KatG and the other mutant proteins led to a decrease in entropy, with $\Delta S^\circ_{\text{rc}}$ values ranging from -18 to -44 J mol$^{-1}$ K$^{-1}$, thus contributing -56 to -188 mV (Table 1) to the negative $E^\circ$ value of these metalloproteins and following the opposite hierarchy compared to enthalpic factors:
Ser335Gly > Trp122Phe > Asp152Ser > Glu253Gln > wild-type KatG (Table 1). By contrast, reduction of ferric His123Gln and Arg119Asn is entropically favored. The corresponding entropic contributions (+513 mV and +763 mV, respectively) partially compensate the enthalpic stabilization of the ferric state in these two mutant proteins.

Figure 4. (A) Temperature dependence of the reduction potential and (B) $E^\circ/T$ versus $1/T$ plots for wild-type Synechocystis PCC 6803 KatG (●) and for its Y249F (○), W122F (●), D152S (●), S335G (●), E253G (●), H123Q (○) and R119N (□) variants. The slope of the plots yields the $\Delta S^\circ_{rc}/F$ and $-\Delta H^\circ_{rc}/F$ values, respectively. Solid lines are least square fits to the data points. Experimental conditions: 0.02 mM and 0.03 mM protein, respectively, in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0.
**Discussion**

The modelled structure of *Synechocystis* KatG is very similar to those of the catalase-peroxidases used as templates, as expected from the high sequence identities (55%-72%) and similarities (78%-88%) existing between the proteins. The structural similarity extends to the residues belonging to the heme cavity (R119, W122, H123 in the distal zone; H290, W341, D402 in the proximal pocket; S335 and W122, Y249, M275, the latter forming the distal covalent adduct) and to the local H-bond network, which involves several conserved water molecules, as well as to the KatG-specific large loop insertions, which build up the access channel to the deeply buried heme and connect the distal and proximal domains.

For the purpose of this work, the comparison of the modelled structures of the ferric and ferrous forms of *Synechocystis* KatG is particularly relevant, since it clearly suggests that electron uptake by the ferric heme causes very limited structural changes, which are mainly limited to a slight rearrangement of the residues and of the water molecules in the distal cavity, inducing a slight increase of its solvent accessibility and of the hydrophilic component of the calculated ASAs (from 52% to 57%). This effect is opposite to that observed in the native form and the six-coordinate adducts of HRP, in which Fe(III) reduction induces a sensible decrease in ASA of the heme group and of the distal residues [18]. These differences are reflected by the opposite sign of the corresponding $\Delta S^\circ_{\text{re}}$ values (Table 1). The positive reduction entropy of HRP has been attributed to a decreased solvent ordering upon reduction, in agreement with a reduced electrostatic interaction of the ferrous heme with the water molecules in the distal cavity as compared to the ferric enzyme and the lower solvent accessibility of the heme group and of the distal residues in the ferrous form is consistent with this picture [18, 19]. On the contrary, the negative $\Delta S^\circ_{\text{re}}$, featured by wild-type *Synechocystis* KatG indicates a reduction-induced increase in the ordering of the water molecules in heme cavity (see below), which fits with the enhanced polarity of the distal site indicated by ASA calculation and with the increased H-bonding interactions with the distal residues.

Table 1 compares the reduction thermodynamics of wild-type *Synechocystis* KatG and of the seven mutant proteins with recent data obtained from other (catalatically-inactive) heme peroxidases, namely horseradish peroxidase isoform C (HRP-C) [16], *Athromyces ramosus* peroxidase (ARP) [17] and myeloperoxidase (MPO) [18]. In
addition, it correlates these data with resonance Raman investigations of the iron-imidazolate bond strength and kinetic parameters of the catalase activity.

Since reduction thermodynamics for metalloproteins in aqueous solution are deeply affected by solvent reorganization phenomena [21-28], the thermodynamic data listed in Table 1, as such, cannot be used to extract information on mutation-induced protein-based effects on the thermodynamics of Fe(III)→Fe(II) reduction. In particular, it is worthy of note that for all the present KatG variants the mutation-induced changes in ΔH°'rc and ΔS°'rc are much larger than the corresponding changes in ΔG°'rc, and therefore in E°' (Table 1). This is typical of the presence of enthalpy-entropy compensation phenomena occurring within a series of homologous species subjected to the same reaction at fixed temperature [21-25, 29-32], according to equation 1 [29-32]:

\[ \Delta H_i = a + b (T \Delta S_i) \]  

(1)

where a and b are constants [21-25, 29-32]. The compensation plot in which the entropic contributions to ΔG°'rc at 298 K (TΔS°'rc) are plotted against the corresponding enthalpic terms (−ΔH°'rc) for wild-type KatG and all the mutants investigated is linear (Figure 5), with a regression coefficient (r²) of 0.999 and a slope close to unit (0.9), indicative of a high degree of compensation. Such H-S compensation indicates that the reaction enthalpies and entropies for the Fe(III)→Fe(II) reduction within the series are dominated by solvent reorganization effects due to reduction-induced changes in the hydrogen bonding network connecting the water molecules within the hydration sphere of the protein [21-25, 29-32]. Since the above solvent reorganization effects are fully compensatory at any temperature (ΔH°'rc(solv) = TΔS°'rc(solv)) [29-32], it turns out that they do not affect the free energy of Fe(III)→Fe(II) reduction. Therefore ΔG°'rc (−nFE°') in native and mutated KatG is totally determined by protein-based effects.

\[ \Delta G°'_{rc} (= -nFE°') = \Delta H°'_{rc(int)} + T\Delta S°'_{rc(int)} \]  

(2)

If we assume, to a first approximation, that the protein-based entropy changes related to differences in conformational degrees of freedom are negligible, then the free energy of Fe(III) to Fe(II) reduction and the reduction potential of the Fe(III)/Fe(II) couple in native and mutated KatGs are determined by the protein-based selective enthalpic stabilization of one of two redox states of the heme:

\[ \Delta G°'_{rc} = \Delta H°'_{rc(int)} \]  

(4)

\[ E°' = -\Delta H°'_{rc(int)}/F \]  

(5)
Hence, any deviation from the perfect H-S compensation, namely any mutation-induced change in $E^\circ$, must arise from protein-based molecular factors, such as alterations in the coordination features of the heme iron and changes in the electrostatics at the interface between the heme and the protein and the solvent (the latter as a bulk effect).

The negligible role of protein-based entropic factors in defining the $E^\circ$ values of the present series of KatG mutants is confirmed by comparison of the 3D structures calculated for the ferric and ferrous forms of the wild-type *Synechocystis* enzyme, which demonstrates that Fe(III) reduction induces very limited changes in the overall protein structure, as indicated by the small rmsd values between the two redox states (see above). The same behavior has been observed in other heme peroxidases, i.e. horseradish peroxidase isoform C (HRP-C) [16], *Athromyces ramosus* peroxidase (ARP) [17] and myeloperoxidase (MPO) [18].

![Figure 5. Enthalpy/entropy compensation plot at 298 K for the reduction thermodynamics of wild-type *Synechocystis* PCC 6803 KatG and its Y249F, W122F, D152S, S335G, E253G, H123Q and R119N variants. Solid line is the least-squares fit to the data points.](image)

KatG, HRP-C and ARP belong to the same peroxidase superfamily [19], that comprises three classes of similar active site architecture. Besides the fully conserved distal side catalytic residues His and Arg (His123 and Arg119, see Figure 2A), all members have the proximal histidine (His259) hydrogen-bonded with an aspartate (Asp402). The latter deprotonates the $N^\delta$ position of the proximal His thereby providing a strong axial ligand with imidazolate character. It increases the electron density at the heme iron and guarantees negative $E^\circ$ values of the couple Fe(III)/Fe(II) that are a precondition for efficient reaction with $H_2O_2$ and for prevention of binding of $^{3}O_2$. Moreover, it helps to
maintain the iron-imidazole bond in optimized strength and geometry. In Class I peroxidases (APXs, CcPs and KatGs) a H-bond between Asp402 and a conserved tryptophan (Trp341) further restricts the position of the proximal His. The importance of this architecture for the redox chemistry of these metalloproteins has been demonstrated by CcP mutants with disrupted His-Asp interaction. Whereas wild-type CcP has been reported to exhibit $E^\circ$ values of the Fe(III)/Fe(II) couple of -194 mV [20] and -182 mV [21], variants with exchanged Asp (Asp235Asn, Asp235Ala, Asp235Glu) had significantly increased (more positive) reduction potentials (-79 mV, -78 mV, -113 mV, respectively) [21]. The corresponding value of native soybean ascorbate peroxidase was reported to be -160 mV [22]. Thus, in Class I peroxidases $E^\circ$[Fe(III)/Fe(II)] values are placed within a relatively small potential region of 66 mV ranging from -226 mV (KatG) to -160 mV (APX). This excludes an important impact of the proximal heme architecture on the mechanism of H$_2$O$_2$ oxidation since both CcP and APX are catalytically inactive. This hypothesis is underlined by the fact that the iron-imidazole bond strength probed by resonance Raman (RR) spectroscopy in the low-frequency region was very similar in wild-type and mutant KatG proteins [23, 24] although the catalytic properties of the investigated variants had a broad variability ranging from wild-type activity to catalytically more or less inactive enzymes (Trp122Phe or Tyr249Phe) (Table 1) [9, 12, 13, 15, 25, 26]. The RR spectrum of wild-type KatG displays two bands at 205 and 253 cm$^{-1}$ that were assigned to two $
u$(Fe-Im) stretching modes [23], with the 203 cm$^{-1}$ band corresponding to a species whose proton resides on the imidazole, while the band at 251 cm$^{-1}$ corresponds to a form where the proton is transferred to the carboxylate. Since all investigated mutants - with the exception of Asp152Ser - showed very similar spectral bands in this region (Table 1) [24], it can be concluded that (i) the mutations had no impact on the proximal heme architecture and the Fe-Im bond strength, and that (ii) observed differences in $E^\circ$ and reduction thermodynamics are related to mutational effects on the distal site and/or substrate channel architecture. In the case of Asp152Ser, the altered low-frequency RR spectrum has been explained by the important role of Asp152 in stabilizing the KatG-typical large loop 1 (LL1) that connects the distal side with the proximal helices E and F [13, 23, 24].

One KatG-typical structural feature, that is absent in CcP and APX, is the distal side covalent adduct Trp122-Tyr249-Met275. Its disruption, that occurs upon exchange of Trp122 (Trp122Phe) or Tyr249 (Tyr249Phe) [13], totally converts a bifunctional KatG into a monofunctional peroxidase [9, 21]. However, it does not modify the standard reduction potential of the heme iron (Table 1), which depends on the protein-based
enthalpic selective stabilization of the ferric form of the enzyme (see above), namely the
coordination features of the heme iron (in agreement with the RR data) and the
electrostatics at the interface between the heme and the protein and the solvent.

Since changes in conformational degrees of freedom of the polypeptide chain upon
reduction can be neglected (see above) [14, 16-18, 27-29] and mainly solvent
reorganization effects contribute to $\Delta S^\circ_{\text{re}}$, the increase of order of solvent upon formation
of ferrous KatG can be related to its channel architecture and H-bond network. In fact,
KatGs have a more deeply buried active site compared to monofunctional (Class III) HRP-
C and the proposed access route for $\text{H}_2\text{O}_2$ is provided by a channel that is similar but
longer and more constricted than that in other heme peroxidases, very similar to
monofunctional catalases [12, 33, 41]. This channel architecture is typical for
catalatically-active enzymes and its manipulation usually decreases the catalase but
increases the peroxidase activity [12, 19, 30-32]. The entropic stabilization of the ferric
form in wild-type KatG fits with a diminished solvent accessibility in bifunctional KatG
compared to monofunctional HRP, which on the contrary features a positive $\Delta S^\circ_{\text{re}}$ value
(Table 1) [19]. The increased loss of entropy upon Fe(III) reduction in both Trp122Phe and
Tyr249Phe mutants compared to the wild-type protein clearly indicates that disruption of
the distal covalent adduct Trp122-Tyr249-Met275 sensibly alters reduction-induced solvent reorganization effects [33]. This agrees with the role played by Trp122 in the
extended network of hydrogen bonds existing in the heme cavity, which includes water
molecules, His123, Arg119 and KatG-specific Asp152 and Glu253 (Figure 2). On the
other hand, Tyr249 belongs to the KatG-typical LL1 loop that stabilizes the heme cavity
architecture. Disruption of the adduct might readjust the LL1 loop and increase the 6-
coordinate low-spin state of Fe(III) [23], decreasing the electrostatic interaction of the
metal ion with the other water molecules in the heme cavity. In any case, these data clearly
show that the competence of a peroxidase to oxidize hydrogen peroxide is not related
directly to the midpoint potential of the heme iron. The role of the unique Trp-Tyr-Met
adduct in catalase activity has been demonstrated recently [10, 11]: it is transiently
oxidized during turnover and supports the rapid oxidation reaction of the second $\text{H}_2\text{O}_2$
molecule as well as the release of dioxygen [10, 11]

Asparate 152 is also fully conserved in KatG and its substitution significantly
reduced the catalase activity with little effect on peroxidase activity (Table 1) [15, 34]. It is
situated in the heme distal cavity at the main access channel entrance. The positioning of
its carboxylate, that is hydrogen-bonded to several water molecules, less than 6 Å from the guanidinium side chain of Arg119 suggests that it might modulate the H₂O₂ entry into the active site, working in concert with Arg119 [34]. Removal of the negative charge made Asp152Ser structurally and spectroscopically similar toCcP [23] and modifies the proximal Fe-Im bond strength most probably due to disabled interaction with Ile248 that is part of LL1 [24]. Simultaneously, H-bonded water molecules might be released upon mutation [23].

In this context, the increased E° of the Asp152Ser mutant compared to the wild-type KatG [ΔE° (E°mut-E°nat) = +18 mV, see Table 1] agrees with the decreased basicity of the proximal His as well as with the deletion of a negative charge near the heme and with a decreased polarity of the distal heme cavity, which reduces enthalpic stabilization of ferric form of the enzyme due to ligand-binding and electrostatic interactions, respectively (Table 1).

Asp152Ser features a ΔS°rc even more negative than wild-type KatG. Upon exchange of the carboxylate group H₂O molecules are released [24] and the continuous solvent matrix is disturbed. As a consequence, reduction-induced solvent reorganization is more restricted than in wild-type KatG. The fact that in a corresponding Burkholderia mutant the K_M for H₂O₂ oxidation was significantly increased [34] underlines the importance of a stable continuum of water molecules in the substrate channel for efficient delivery and binding of hydrogen peroxide.

Mutants of serine 335 (Ser315 in Mycobacterium tuberculosis) play a key role in isoniazide-resistant strains in treatment of tuberculosis. As observed with the Trp122Phe and Tyr249Phe variants, the Ser335Gly mutation does not influence the E° value, but induces a more negative ΔS°rc compared to wild-type KatG (Table 1), clearly demonstrating it does not modify the protein-based enthalpic stabilization of the ferric enzyme, although it sensibly alters the reduction induced solvent-reorganization. Together with Asp152, Ser335 is located at the narrowest part of the substrate channel close to the heme edge [35]. Its exchange by glycine should open the access to the heme active site for the substrate molecules. Indeed, binding of O₂ to ferrous Ser335Gly (as well as to ferrous Arg119Asn and His123Gln, see below) has been seen in the initial electrochemical studies and could only be hindered by addition of small amounts of laccase and catalase into the protein solution. On the other hand Ser335 is not involved in maintenance of the solute matrix [35] and thus its exchange should not directly have an impact on its rigidity. This is
reflected by its almost intact catalatic properties (Table 1). However, disruption of the hydrogen bond between the side chain hydroxyl group of Ser335 and the carboxyl group of one of the pyrrole propionates in observed in Mycobacterium KatG(Ser315Gly) [36], might diminish the electrostatic interaction of the metal ion with the solvent matrix thus being responsible for the observed negative $\Delta S^{\circ}$ values.

Although loss of the carboxylate group at the entrance of the access channel in Glu253Gln significantly alters the bifunctionality of KatG [12], its $E^{\circ}$ and reduction thermodynamics were nearly coincident with that of the wild-type protein (Table 1). This fits with recently published data that demonstrated neither a change in the spin state nor in the Fe-Im bond strength in Glu253Gln [12] and with the small influence that deletion of surface charges generally have on $E^{\circ}$ in redox metallo-proteins [51-53]. These data clearly suggest that exchange of the carboxylate group has neither an impact on the protein-based molecular factors which define the $E^{\circ}$ of the iron ion nor on solvent reorganization effects at least in the main (inner) part of the access channel. Its role in catalysis (Table 1) might be in attracting and orienting the incoming H$_2$O and H$_2$O$_2$ dipoles [12].

Compared to the so far discussed KatG variants, exchange of the fully conserved distal pair His-Arg significantly modified the redox behavior of the heme iron. In both Arg119Asn and His123Gln mutants the midpoint potential shifted 79 mV and 93 mV, respectively, to more negative (HRP-like) values (Table 1). In contrast to the other mutants investigated in this work, exchange of both His123 and Arg119 affected both the catalatic and the peroxidatic reaction underling the importance of His and Arg in the heterolytic cleavage of hydrogen peroxide [37].

Interestingly, the behavior of the His123Gln KatG mutant coincides with that of His52Gln and His52Asn CcP mutants [55], which feature $E^{\circ}$ values 35 and 70 mV more negative than the wild-type enzyme, respectively, and stabilize the ferric form of CcP more effectively than the His52Glu and His52Asp mutations, in which a negative charge has been inserted in the distal heme cavity [55]. Ion pairing of the carboxylate sidechains with the distal arginine, mutation-induced alterations in the hydrogen bonding network and in the heme legation have been indicated as the possible causes of the above effect [55].

RR spectroscopy indicates that in the His123Gln and Arg119Asn KatG variants the proximal heme architecture is only marginally weakened (Table 1) [23], clearly demonstrating that their lower reduction potentials is not the consequence of an increased interaction with the axial histidine. On the other hand, the above mutations open the heme
distal cavity, inducing a deep reorganization of the hydrogen bonding network and increasing the accessibility of solvent molecules to the metal ion. Opening of the heme cavity in His123Gln and Arg119Asn is evident by the fact that both proteins were more pronounced to dioxygen binding to their ferrous forms than wild-type KatG. A similar effect has been described for HRP and CcP, where mutation of distal His and Arg significantly enhanced the binding rate for \( \text{O}_2 \) [38]. So special care had to be taken in the spectroelectrochemical experiments in order to maintain anaerobic conditions and prevent \( \text{O}_2 \) binding (i.e. compound III formation).

Opening the heme cavity increases the solvent accessibility to the metal center and, consequently, the polarity of the distal heme cavity, electrostatically stabilizing the ferric form of the enzyme compared to the wild-type enzyme. Moreover, an increased polarity of the heme distal site would partially quench the effect of the exchange of the positively charge arginine with the neutral asparagine on \( E^{\circ'} \), explaining, at least partially, the smaller effect of the Arg119Asn mutation has on the reduction potential.

An increased solvent accessibility to the metal center, induced by the mutation-induced opening the heme cavity agrees with the positive \( \Delta S^{\circ'}_{\text{rc}} \) values featured by both the His123Gln and Arg119Asn mutants, which are typical for metalloproteins with solvent exposed central ions [16, 27-29]. Exchange of both His and Arg furthermore promoted reduction induced solvent reorganization effects since both are essential components of the H-bond network. This is also obvious by an increased fraction of 6-coordinated high-spin heme in both mutant proteins [23].

Summing up, this work describes the redox thermodynamics of bifunctional catalase-peroxidase variants that carry mutations at sites known to be important in catalysis as well as in stabilization of the KatG-typical H-bonding network. Exchange of the catalytic distal residues His and Arg completely reorganizes the heme cavity architecture and accessibility and dramatically modifies the redox properties of the corresponding mutants. In other (catalytically active and inactive) mutants the midpoint potential was very similar to that of wild-type KatG though the relative contributions of \( \Delta H^{\circ'}_{\text{rc}} \) and \( \Delta S^{\circ'}_{\text{rc}} \) to \( E^{\circ} \) were different and compensated each other. This includes also variants without the KatG-specific Trp-Tyr-Met adduct that is essential for \( \text{H}_2\text{O}_2 \) oxidation. Enthalpic and entropic contributions to \( E^{\circ} \) reflect mutation-mediated alterations of charge and polarity of the distal heme cavity as well as of the rigidity and continuity of
the solvent molecules in the active site and substrate channel. Disruption of the solvent matrix in the constricted access channel of KatG retards oxidation of H$_2$O$_2$ but (with exception of distal His-Arg) not its reduction, i.e. heterolytic cleavage.

**Materials and Methods**

Mutagenesis, expression and purification of wild-type KatG and KatG variants from *Synechocystis* PCC 6803 were described previously [9, 12, 15, 25, 26]. All chemicals were reagent-grade.

Spectroelectrochemistry. All experiments were carried out in a homemade OTTLE cell [16, 27, 28]. The three-electrode configuration consisted of a gold minigrid working electrode (Buckbee-Mears, Chicago, IL), a homemade Ag/AgCl/KCl sat microreference electrode, separated from the working solution by a Vycor set, and a platinum wire as the counter electrode. The reference electrode was calibrated against a saturated calomel electrode before each set of measurements. All potentials are referenced to the SHE. Potentials were applied across the OTTLE cell with an Amel model 2053 potentiostat/galvanostat. The function of the OTTLE cell was checked by measuring the reduction potential of yeast iso-1-cytochrome c in conditions similar to those used in the present work. The $E^\circ$ value corresponded to that determined by cyclic voltammetry (+260 mV). Constant temperature was maintained by a circulating water bath, and the OTTLE cell temperature was monitored with a Cu-costan microthermocouple. UV-vis spectra were recorded using a Varian Cary C50 spectrophotometer. Since some of the mutants were more prone to O$_2$ binding to their ferrous form than wild-type KatG, besides flushing with argon, small amounts of laccase and catalase were present in the cuvettes in these cases.

Variable temperature experiments were performed using a non-isothermal cell configuration. The temperature of the reference electrode and the counter electrode was kept constant, whereas that of the working electrode was varied. Factorization of enthalpic and entropic components, was possible by calculating $\Delta S^{\circ}_{rc}$ from the slope of the plot $E^{\circ}_{rc}$ versus temperature, whereas $\Delta H^{\circ}_{rc}$ could be obtained from the Gibbs-Helmholtz equation, $\Delta G^{\circ}_{rc} = \Delta H^{\circ}_{rc} - T\Delta S^{\circ}_{rc} = -nF E^{\circ}$, thus from the slope of the plot of $E^{\circ}/T$ versus $1/T$ [29]. The Faraday constant $F$ is 96485.31 C mol$^{-1}$, and $n$ represents the number of electrons transferred by the redox couple. All experiments were carried out under Argon over the 10-30°C (Ser335Gly), 10-35°C (Arg119Asn), 15-35°C (Trp122Phe, His123Gln), 15-40°C (Asp152Ser, Glu253Gln) range using 1 mL samples containing protein (concentrations see below) in 50 mM phosphate buffer, pH 7.0, containing 10 mM NaCl, in the presence of 84
various mediators: methyl viologen (mediator A) and a solution of lumiflavine-3-acetate, indigo disulfonate, phenazine methosulfate, and methylene blue (mediators B). In detail, protein and mediator concentrations were as follows: Asp152Ser (0.02 mM), 0.7 mM mediator A and 3 µM mediators B; Glu253Gln (0.035 mM), 0.7 mM mediator A and 3.5 µM mediators B; Trp122Phe (0.016 mM), 0.28 mM mediator A and 2 µM mediators B; His123Gln (0.025 mM), 0.2 mM mediator A and 2.5 µM mediators B; Arg119Asn (0.02 mM), 0.2 mM mediator A and 2.5 µM mediators B. In order to prevent binding of oxygen to the Fe(II) form catalase from bovine liver and laccase from *Rhus vernicifera*, 0.5 µM each, were added to the sample containing 0.024 mM Ser335Gly, 0.55 mM mediator A and 2 µM mediators B.

Nernst plots consisted of at least six points and were invariably linear, with a slope consistent with a one-electron reduction process.

**Molecular modelling. Model building.** The sequence of the catalase-peroxidase from *Synechocystis* sp. PCC 6803 (UniProtKB entry P73911) was extracted from the UniProtKB/Swiss-Prot database [61] (www.expasy.org), with the accession code P73911. A FASTA [62] search performed against the Protein Data Bank [63] identified the proteins with the highest sequence identity (id) and similarity (ss) to the target sequence: the catalase-peroxidases from *Burkholderia Pseudomallei* (PDB entry: 2FXH, chain A; id=60%, ss=81%), *Mycobacterium Tuberculosis* (PDB entry: 2CCA, chain B; id=55%, ss=78%), *Haloarcula Marismortui* (PDB entry: 1ITK, chain B; id=55%, ss=80%) and *Synechococcus sp.* (PDB entry: 1UB2; id=72%, ss=86%). In the cases when more than one 3D structure was available, a few structural criteria (i.e. best resolution, pH around 7, structural completeness, highest sequence identity to the target) were used to select the best representative protein structure to be used as a template.

The target sequence was then aligned to the sequences of the four templates. The multiple alignment used for modelling (see Supplemental Figure S1) was performed with the software CLUSTALW [64] (www.ebi.ac.uk).

Modelling of the wt catalase-peroxidase from *Synechocystis* sp. PCC 6803 was performed with the software MODELLER v. 8.2 [65]. Five model structures were generated and subsequently i) assessed against the available structural experimental information and ii) evaluated from a stereochemical and geometrical perspective with the aid of the tools implemented in the software packages: MODELLER [65], PROCHECK [66], QUANTA [67]. The values of the objective function output by MODELLER [65] together with the values of the global G-factors and of the Ramachandran residue
distribution from PROCHECK [66], calculated for the models and the template structures, are reported in Supplemental Table S1 (in the Supplementary materials). The modeled structure with the best fitting to the experimental data and the best technical evaluation was then selected and used for further work. Secondary structure prediction was performed with the PredictProtein service (http://www.predictprotein.org/) [68].

Model refinement. The main structural problems are related to the difficulty of reproducing in the 3D model the correct geometry of the adduct built by residues W122-Y249-M275, which are covalently connected. The geometric distortion is due to the local structural differences found in the four 3D structures used as templates. The correct covalent geometry of the adduct was reproduced by manually rotating the side chains of the three residues involved on the basis of the local structure of the catalase-peroxidase from *Synechococcus sp.* (1UB2), which is the protein with the highest sequence identity to the target protein.

The selected modelled structure was then optimized with the GROMACS suite [69], using the GROMOS'96 force field [70]. The crystallographic water molecules in the cell unit of the catalase-peroxidase from *Synechococcus sp.* (1UB2) were translated to the *Synechocystis sp.* PCC 6803 model, to correctly reproduce the active site local environment. The system was then solvated in a cubic water cell with sides extending 15 Å from the protein boundary. All water molecules were treated explicitly with the SPCE model. Short range interactions were treated with the Lennard-Jones potential using a cut-off of 14 Å, while long range interactions were calculated using the PME algorithm within a radius of 10 Å and an interpolation order of 6. All covalent distances were restrained with the LINCS algorithm [71]. 31 and 32 Na⁺ ions were added respectively to the water bulk of the ferric and the ferrous proteins, respectively, to assure system neutrality. The optimization protocol consisted of a preliminary Steepest Descent minimization until energy converged to 1000 kJ/mol, followed by Conjugate Gradients until energy convergence to 1000 kJ/mol was reached. During the calculations, the correct geometries of both the covalent adduct and the heme-H190 system were preserved by adding a few harmonic restraints to their bond length.

Calculation of protein structural parameters. Accessible surface areas were computed for both the reduced and the oxidized 3D models of the catalase-peroxidase from *Synechocystis sp.* PCC 6803. ASAs were computed on the heme group and on the redox active site (heme plus residues R119, W122, H123, Y249 and D152) using the software package QUANTA [67]. The calculations, based on the Richards method [72],
used an all-atoms parameterization for the protein and a conventional sphere of 1.4 Å as the probe simulating a solvent (water) molecule.

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**Supplementary Materials**

**Supplemental Table S1.** Technical checks report. *Parameters of the Ramachandran residue distribution: percentage of residues with correct (core), allowed (all), generously allowed (gener) or disallowed (disall) $\phi/\psi$ conformation.**

**Geometric (G) factors: evaluation of the dihedral conformations (dihedrals) and of the covalent bonds (cov) of all the protein residues and global evaluation (overall) of the protein geometry (values below -0.5: unusual; values below -1.0: highly unusual).***

**MOLPDF: value of the MODELLER objective function, which estimate the content of structural restraint violation in a modelled structure.**

<table>
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<th>MOLPDF***</th>
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Supplemental Figure S1. Multiple sequence alignment of the target sequence (P73911) to the catalase-peroxidase sequences of *Burkholderia pseudomallei* (2FXH_A), *Mycobacterium tuberculosis* (2CCA_B), *Halocarcula marismortui* (2FXH_A), and *Synechococcus sp.* (1UB2). The sequence conservation (conservation), the secondary structure prediction (predSS), and the secondary structure of the model (model secstr) are also reported: "\*\*\*" = identical amino acids; ".\." = low residue conservation; H = α-helix; E = β-strand; 3 = 3-turn; 4 = 4-turn. Color code: red = non resolved amino acids; white = residues in the distal sites; pink = covalent adduct; yellow = residues identifying the access channel to the active site; cyan = Fe coordinating histidine; grey: residues in the proximal site; blu = distal histidine; green = distal arginine.
Supplemental Figure S2. (A) Electronic absorption spectra of the *Synechocystis* KatG variant KatG(W122F) obtained at various applied potentials at 25°C. The inset depicts the Nernst plot, where \( X \) represents \( \frac{(A_{\text{ox}} \text{max} - A_{\text{red}})}{(A_{\text{red}} \text{max} - A_{\text{red}})} \) with \( \lambda_{\text{ox}} = 409 \text{ nm} \) and \( \lambda_{\text{red}} = 430 \text{ nm} \). The absorbance at 430 nm was used for calculations, while the absorbance maximum of the reduced species is at 426 nm. Conditions: 0.016 mM in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0. (B) Electronic absorption spectra (at 25°C) and Nernst plot for the variant KatG(H123Q). \( X \) represents \( \frac{(A_{\text{ox}} \text{max} - A_{\text{red}})}{(A_{\text{red}} \text{max} - A_{\text{red}})} \) with \( \lambda_{\text{ox}} = 406 \text{ nm} \) and \( \lambda_{\text{red}} = 439 \text{ nm} \). The absorbance maximum of the reduced species is at 429 nm. Conditions: 0.025 mM in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0. (C) Electronic absorption spectra (at 25°C) and Nernst plot for the variant KatG(R119N). \( X \) represents \( \frac{(A_{\text{ox}} \text{max} - A_{\text{red}})}{(A_{\text{red}} \text{max} - A_{\text{red}})} \) with \( \lambda_{\text{ox}} = 407 \text{ nm} \) and \( \lambda_{\text{red}} = 439 \text{ nm} \). The absorbance maximum of the reduced species is at 424 nm. Conditions: 0.02 mM in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0. (D) Electronic absorption spectra (at 25°C) and Nernst plot for the variant KatG(S335G). \( X \) represents \( \frac{(A_{\text{ox}} \text{max} - A_{\text{red}})}{(A_{\text{red}} \text{max} - A_{\text{red}})} \) with \( \lambda_{\text{ox}} = 409 \text{ nm} \) and \( \lambda_{\text{red}} = 431 \text{ nm} \). The absorbance maximum of the reduced species is at 425 nm. Conditions: 0.024 mM in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0. (E) Electronic absorption spectra (at 25°C) and Nernst plot for the variant KatG(E253Q). \( X \) represents \( \frac{(A_{\text{ox}} \text{max} - A_{\text{red}})}{(A_{\text{red}} \text{max} - A_{\text{red}})} \) with \( \lambda_{\text{ox}} = 406 \text{ nm} \) and \( \lambda_{\text{red}} = 437 \text{ nm} \), where the reduced species exhibits its absorbance maximum. Conditions: 0.035 mM in 50 mM phosphate buffer and 10 mM NaCl at pH 7.0.
chapter five

Putative substrate binding site(s) in bifunctional catalase-peroxidases

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Unpublished Data
Putative substrate binding site(s) in bifunctional catalase-peroxidases

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Introduction

Catalase-peroxidases (KatGs), present in prokaryotes and fungi, are unique bifunctional enzymes since they exhibit a predominant catalase activity together with a substantial peroxidatic activity with broad specificity. The latter becomes significant in the presence of a suitable one-electron donor and at low (i.e. submillimolar) levels of hydrogen peroxide. However, neither the in vivo peroxidatic substrate nor its binding site have been identified, leaving the actual role of the peroxidatic reaction undefined. Though being homologous to cytochrome c peroxidase (CcP) and ascorbate peroxidase (APX), KatG is the only heme peroxidase that is able to dismutate hydrogen peroxide (2 \( \text{H}_2\text{O}_2 \rightarrow 2 \text{O}_2 + 2 \text{H}_2\text{O} \)) by a non-scrambling mechanism (1) with rates similar to monofunctional catalases. Together with ascorbate peroxidases, present in chloroplastic organisms, and cytochrome c peroxidase, found mainly in mitochondrial organisms, KatGs constitute class I of the plant, fungal, and bacterial heme peroxidase superfamily (2-4). The four available crystal structures of KatG enzymes from *Haloarcula marismortui* (Protein Data Bank code 1ITK), *Burkholderia pseudomallei* (code 1MWV), *Mycobacterium tuberculosis* (code 1SJ2), and *Synechococcus* PCC 7942 (code 1UB2) (5-8) revealed that the heme pocket contains catalytic residues virtually identical to those of other peroxidases belonging to class I. In particular, the distal and proximal heme pockets contain the amino acid triads His-Arg-Trp and His-Trp-Asp, respectively. However, features unique to catalase-peroxidases were also found; like the unusual covalent adduct consisting of the distal side residues Trp122, Tyr249, and Met275 (in *Synechocysits* numbering) (5-8), and an arginine (Arg439) that seems to act as a molecular switch with the guanidinium group being either in association with the tyrosinate ion of the adduct (Y-conformation) or being shifted to a region containing two other arginine residues (R-conformation) (Figure 1A) (3, 6).

The catalatic activity is closely related with the funnel-shaped main access channel that leads to heme \( b \), which is more deeply buried than in (monofunctional) peroxidases.
Moreover, the channel is longer and restricted near Asp152 allowing only small molecules to enter the heme cavity. An ordered continuum of water molecules and rigid H-bonding network is important for the H$_2$O$_2$ oxidation reaction. Whenever residues in the active site that are part of this network were mutated and hence the hydrogen-bonds disrupted, the catalatic activity was heavily reduced, while the peroxidase activity remained unaffected.

As mentioned above the binding and oxidation site of conventional peroxidase substrates like guaiacol and ABTS as well as the nature of the endogenous one electron (AH$_2$) donor of catalase-peroxidases is unknown (H$_2$O$_2$ + 2 AH$_2$ → 2 H$_2$O + 2 *AH). An access route approximately in the plane of the heme, normally found in (monofunctional) peroxidases is blocked by loops in KatG. However, from structural analysis of *Burkholderia* KatG Carpena et al. (6) described another access route providing direct access to the core of the protein. It is located in a region that encompasses two structural features with possible functional significance. One is the presence of a cleft with a U-shaped region aligned with polar residues (Figure 1B). The second feature is related with a putative role of the “flipping” Arg439 (Figure 1A). In one conformation Arg439 is located on the surface at the bottom of the “U”, whereas in the other conformation it increases the depth of the cleft and reduces the positively charges on its surface. Such a striking and well-defined cavity, combined with the potential for functional changes through the simple movement of a side-chain (Arg439), begs the suggestion that this cavity might be the binding site for a peroxidase (i.e one-electron donating) substrate.

In order to test whether the U-shaped cleft functions as peroxidase substrate binding site, we have performed a sequence alignment of 156 genes encoding both prokaryotic and eukaryotic KatGs (Figure 2) in order to see conservation of relevant amino acids. Finally, five residues were found to be conserved in almost all *katG* genes available so far in public databases, namely Asn278, Glu281, Lys435, Trp459 and Arg509 (*Synechocystis* numbering). The variants Asn278Asp, Glu281Asp, Glu281Gln, Lys435Gln, Lys435Arg, Trp459Phe, and Arg509Lys were recombinantly expressed in *E. coli* and the overall catalase and peroxidase (guaiaicol and ABTS) activity was investigated. Furthermore, we have tested the reaction of the ferric proteins with both hydrogen peroxide and peroxoacetic acid in order to see whether the spectral and kinetic features follow the known pattern of wild-type KatGs (9). Data obtained for the variant Arg439Ala are also presented for comparative studies.
Figure 1. Detailed view of conserved amino acid residues in the U-shaped region of *Burkholderia pseudomallei* KatG. Figures were constructed using PyMol. (A) KatG-specific residues in the vicinity of the heme including the distal site covalent adduct Trp-Tyr-Met and the "flipping" arginine, which can adopt two conformations (Y and R) (corresponding *Synechocystis* residues are given in brackets). Additionally, the position of the residues Asn267(278), Glu270(281), Lys422(435), Trp446(459), and Arg497(509) that are highly conserved residues in U-shaped region. (B) Slab view showing the U-shaped region with the amino acids Asn267 (red), Glu270 (yellow), Lys422 (blue), Trp446 (orange), Arg497 (cyan), and Arg426 (magenta), the covalent adduct (grey), and the heme (green). (B) U-shaped region in *Burkholderia pseudomallei* KatG constructed using the PyMol plugin CASTp with the same coloured residues as in (A) and (B).
Figure 2. Selected parts of the multiple sequence alignment of 156 KatGs. 45 complete bacterial sequences including residues that contribute to the structural composition of the putative substrate binding site. Abbreviations of analyzed sequences correspond to PeroxiBase nomenclature (http://peroxibase.isb-sib.ch/ for all details)*. Arrows indicate the residues mutated in the present study. (A) Asn278, Glu281. (B) Lys435, Arg439.

**Materials and Methods**

**Reagents** – Standard chemicals and biochemic als were obtained from Sigma at the highest grade available.

**Mutagenesis** – A pET-3a expression vector containing the cloned catalase-peroxidase gene from the cyanobacterium *Synechocystis* PCC 6803 (10) was used as the template for PCR. Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described previously (11). Unique restriction sites flanking the region to be mutated were selected. The flanking primers for the *Synechocystis* KatG variants Asn278Asp, Glu281Asp, and Glu281Gln were 5′-AAT GAT CAG GTA CCG GCC AGT AAA TG-3′ (containing a KpnI restriction site) and 5′-AGT GCA GAC TAG TTC GGA AAC G-3′ (containing a SpeI restriction site). The mutant primer with the desired mutation changing Asn278 to Asp and a silent mutation introducing a PauI restriction site was 5′-TTT GC\_CGC\_ATG GCC ATG GAC GAG GAA AC-3′ and the internal 3′-primer with the same introduced restriction site was 5′-CAT\_CGC\_AAA GGT AGT GGT AGT G-3′ (with point mutations in italics and introduced restriction sites underlined). To substitute Glu281 with Asp and Gln, respectively, the used internal 5′-primer with a PstI restriction site introduced by silent mutation was 5′-CTT ACT GCA GGG GGG CAC AC-3′ and the mutant primers with the same introduced restriction site were 5′-CC CCC\_TGC\_AGT AAG GGC AAC GGT ATC CTC GTC ATT C-3′ and 5′-CC CCC\_TGC\_AGT AAG GGC AAC GGT TTG CTC GTC ATT C-3′, respectively.

For the variants Lys435Gln, Lys435Arg, and Trp459Phe the flanking primers were 5′-GG CAC CCG GAT CCT TTA TG-3′ (containing a BamHI restriction site) and 5′-AGT GCA GAC TAG TTC GGA AAC G-3′ (containing a SpeI restriction site). Following internal primers were used to substitute Lys435 with Gln and Arg, respectively: 5′-GCC AA4 GCT TGG TTT CAA CTA ACT CAC-3′ changed Lys435 to Gln, 5′-CC AA4 GCT TGG TTT AGA CTA ACT CAC-3′ changed Lys435 to Arg, and 5′-A CCA AGC TTT GGC AAA TAC-3′ a 3′-primer used for both KatG variants (underlined the introduced HindIII restriction site). To substitute Trp459 with Phe the following internal primers containing an AccIII restriction site were used: 5′-T GG\_CCG\_GAT GTG CCC CAG GAA GAT TTA ATT TTT CAA GAC CCC-3′ and 5′-AC ATC CGG\_ACC AAG GTA AC-3′. The flanking primers 5′-GG CAC CCG GAT CCT TTA TG-3′ (containing a BamHI restriction site) and 5′-GCC CCT AGG GAG ATC AAA CCG G-3′ (containing an AvrII restriction site) and the internal primers, both with an introduced BbeI restriction
the latter containing the desired mutation, were used to create the KatG variant Arg509Lys.

Expression and Purification – The mutant recombinant catalase-peroxidases were expressed in *Escherichia coli* BL21(DE3)pLysS and purified as described previously (10, 12) using the same conditions as for the wild-type enzyme.

Circular dichroism – CD studies were carried out using a PiStar-180 spectrometer from Applied Photophysics. Far-UV (190–250 nm) experiments were performed using a protein concentration of 2 µM and a cuvette with 1 mm path length. A good signal-to-noise ratio in the CD spectra was obtained by averaging 3 scans (step, 1 nm; bandwidth, 3 nm; default sampling size, 150 000; maximum sampling size, 500 000). The protein concentration was calculated from the known amino acid composition and absorption at 280 nm according to Gill and Himmel (13).

Steady-state kinetics – Catalase activity was determined polarographically in 50 mM phosphate buffer using a Clark-type electrode (YSI 5331 oxygen probe) inserted into a stirred water bath (YSI 5301B). All reactions were performed at 30°C and started by addition of KatG. One unit of catalase is defined as the amount that decomposes 1 µmol of H₂O₂/min at pH 7.0 and 30°C. To cover the pH range 3.0–9.0, 50 mM citrate/phosphate and 50 mM Tris-HCl buffer were used.

Peroxidase activity was monitored spectrophotometrically using 1 mM H₂O₂ and 5 mM guaiacol (ε₄₇₀ = 26.6 mM⁻¹ cm⁻¹) or 2.5 mM H₂O₂ and 0.3 mM ABTS (ε₄₁₄ = 36.8 mM⁻¹ cm⁻¹). Alternatively, 1 mM peroxoacetic acid (PAA) and 5 mM guaiacol were used. One unit of peroxidase is defined as the amount that oxidizes 1 µmol of electron donor/min at pH 5.5 and 25°C. To cover the pH range 3.0–9.0 same buffers as for catalatic activity were used.

Transient-state kinetics – Transient-state measurements were made using a SX.18MV stopped-flow spectrometer (Applied Photophysics Ltd.) equipped with a 1 cm observation cell. Calculation of pseudo first-order rate constants (kobs) from experimental traces at the Soret maximum was performed with a SpectraKinetic work station (Version 4.38) interfaced to the instrument. The substrate concentrations were at least five times that of the enzyme to allow determination of pseudo first-order rate constants. Observed pseudo-first-order rates were plotted as a function of substrate concentration to obtain apparent second-order rate constants for compound I formation. To follow spectral transitions, a PD.1 photodiode array accessory (Applied Photophysics Ltd.) connected to
the stopped-flow device together with XScan diode array scanning software (Version 1.07) was utilized. The kinetics of oxidation of ferric catalase-peroxidase to compound I by peroxoacetic acid were followed in the single mixing mode. Catalase-peroxidase and peroxoacetic acid were mixed to give a final concentration of 2 µM enzyme and 20–200 µM PAA. The first data point was recorded 1.5 ms after mixing, and 2000 data points were accumulated. Sequential mixing stopped-flow analysis was used to monitor compound I reduction by one-electron donors. In the first step, the enzyme was mixed with peroxoacetic acid, and after a defined delay time, the formed compound I was mixed with ascorbate as the electron donor.

All stopped-flow measurements were conducted at 25°C in 50 mM phosphate buffer, pH 7.0, and at least three determinations were performed per substrate concentration.

**Results**

*Spectral properties –* The UV-Vis absorption spectra of wild-type KatG and variants exhibited the typical bands of a heme \( b \) containing peroxidase. The Soret band varied between 406 and 407 nm and the two bands in the visible region were at 510 and 637 nm (CT).

The far-UV (190-250 nm) CD spectrum is a sensitive probe of protein secondary structure. The CD spectra of all variants (data not shown) were similar to that of wild-type KatG and showed typical features of an \( \alpha \)-helical protein structure with the 222 and 208 nm (negative) dichroic bands. Therefore mutations did not induce changes in the overall secondary structure and if conformational changes did occur, they must be very localized and minimal.

*Overall bifunctional activity –* The kinetic parameters for the catalase and peroxidase activities of wild-type KatG and the investigated variants are summarized in Table 1. Additionally, data obtained upon exchange of the “flipping” arginine with alanine (Arg439Ala) (14) are presented. Wild-type KatG exhibits an overwhelming catalase activity with an apparent \( k_{\text{cat}} \) of 3500 s\(^{-1}\) (11). Significant changes in the catalatic activities were only observed for the variants Lys435Arg and Lys435Gln. The \( k_{\text{cat}} \) values of these mutants were 70 and 49%, respectively, of that of wild-type KatG. Similar turnover numbers, compared to wild-type, were obtained for the variants Asn278Asp, Glu281Asp, and Glu281Gln, whereas Trp459Phe and Arg509Lys showed slightly higher turnover numbers. Regarding the apparent \( K_M \) value in catalase activity, Lys435Gln is the only
variant analyzed within this work, that exhibits diminished affinity with H$_2$O$_2$ (6.1 mM) compared to the wild-type protein (4.1 mM).

In WT KatG, the pH profile of the catalase activity has a sharp maximum at pH 6.5 (Figure 3, black line) (15). The same behaviour was shown for the other variants (data not shown), except of the Lys435Gln variant. The pH dependence of the catalatic activity in Lys453Gln shows a plateau between pH 5-6 and a maximum at pH 7.5 (Figure 3, gray line).

Figure 3. pH-dependence of the catalatic activity in wild-type KatG (black line) and Lys435Gln (gray line). The catalatic activity was determined polarographically. Conditions were as follows: 5 mM hydrogen peroxide, 50 mM citrate/phosphate or 50 mM Tris-HCl buffer at 30°C.

The effect of the performed mutations on the peroxidase activity (Table 1) was small. In some variants the peroxidase activity was even slightly enhanced. Two substrates, namely guaiacol and ABTS, were used as artificial one-electron donors. No significant differences between wild-type and most variants using guaiacol in combination with hydrogen peroxide as the peroxidase substrate were observed. Only Lys435Arg showed a decrease in activity of about 80%, whereas the variants Arg509Lys and Arg439Ala (14) exhibited a 1.5 and 2-fold increase in peroxidase activity. With ABTS most of the variants exhibited slightly reduced or wild-type like activities. Lys435Gln and Arg439Ala had an increased capacity to oxidize ABTS.

In order to exclude the interference of the overwhelming catalatic activity with the peroxidase activity, the guaiacol assays were also performed using peroxyacetic acid instead of hydrogen peroxide. Under these conditions not only Lys435Arg but also Glu281Asp, Lys435Gln, and Arg439Ala (14) exhibited decreased peroxidase activity (Table 1). However, the effect was not very significant thereby excluding an important role of the mutated residues in binding and oxidation of either ABTS or guaiacol. This is also
underlined by the fact that the pH maximum of peroxidase activity determined with ABTS as one-electron donor was at pH 5.5 for wild-type KatG as well as for all mutants investigated in the present study (data not shown).

**Compound I formation with peroxoacetic acid** – Because of the overwhelming catalatic activity in catalase-peroxidases the formation of compound I mediated by hydrogen peroxide cannot be followed spectroscopically. As a consequence, organic peroxides (e.g. peroxoacetic acid) have to be used to monitor the formation of an oxoiron(IV) porphyrin radical compound I (Por$^{•+}$Fe$^{IV}$=O), which (at least in most monofunctional peroxidases) is typically characterized by a 40-50% hypochromicity in the Soret region. Compound I formation from ferric *Synechocystis* wild-type KatG with peroxoacetic acid is characterized by a 45% hypochromicity at 407 nm and the appearance of two prominent peaks around 600 and 650 nm (11). Similar results were obtained for the variants investigated in the present work. However, the observed hypochromicity at the Soret region was less pronounced (27–36%) compared to the wild-type (data not shown).

The reaction of wild-type KatG with peroxoacetic acid followed at 407 nm was monophasic and could be fitted using a single exponential equation. Observed pseudo first-order rates ($k_{obs}$) plotted as a function of peroxoacetic acid concentration resulted in an apparent second-order rate constant ($k_{app}$) of \((3.9 \pm 0.3) \times 10^4\) M$^{-1}$s$^{-1}$ at pH 7.0 (11). No significant differences were seen in the reactions of the variants with peroxoacetic acid. The observed reactions were monophasic and could be fitted using a single exponential equation. The resulting $k_{obs}$ values plotted against peroxoacetic acid concentration yielded apparent second-order rate constants ranging from \(1.9 \times 10^4\) M$^{-1}$ s$^{-1}$ to \(5.1 \times 10^4\) M$^{-1}$ s$^{-1}$ at pH 7.0 (Table 1). Only in Arg439Ala the reaction was significantly increased by a factor of 18 (2).

**Reaction of ferric KatG with hydrogen peroxide** – Because of the overwhelming catalase activity only the ferric form of wild-type KatG can be monitored when mixed with moderate levels of hydrogen peroxide. Addition of a large excess of hydrogen peroxide, however, immediately forms a redox intermediate with a red-shifted Soret band (414 nm) and two new peaks at higher wavelength (520 and 580 nm) at intermediate pH values (9). This pH dependent species was formed immediately after mixing (1.3 ms) and was the dominant redox intermediate as long as hydrogen peroxide was available.
Table 1. Steady-state and pre-steady-state kinetic parameters of catalase and peroxidase activity of wild-type KatG from *Synechocystis* and selected variants. For comparative purposes published data from Arg439Ala are also presented.

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</table>


*Kinetic data were extracted from spectral transitions using ProK software from Applied Photophysics.*
Like in wild-type KatG all the variants did not allow monitoring of compound I formation or did not show any spectral transitions upon using moderate excess of hydrogen peroxide. However, high excess of hydrogen peroxide led to similar spectral transitions as observed in the wild-type enzyme at pH 8.5 (9). As a representative example the spectral transitions of the ferric Arg509Lys mixed with millimolar H$_2$O$_2$ at pH 7.0 is shown in Figure 4. The reaction started with a 1.3 ms spectrum with peaks at 414 nm and 520 nm and ended with the Soret band at 407 nm and reappearance of the CT band at 637 nm after depletion of hydrogen peroxide. The variants Glu281Asp/Gln, Lys435Arg, Trp459Phe, and Arg509Lys showed similar spectral transitions upon mixing of 2 µM enzyme with 2500-fold excess of hydrogen peroxide (data not shown). Different from the other variants but similar to wild-type KatG at pH 5.6 (9) were the spectral transitions observed in Lys435Gln. Because of the low catalase activity only a 100-fold excess of hydrogen peroxide was necessary to get a reaction intermediate with peaks at 410, 545, and 578 nm at pH 7.0 (Figure 5A, black line). Upon using a higher excess of hydrogen peroxide (500-fold) the Soret maximum was red-shifted to 414 nm (Figure 5A, bold line).

Interestingly and different from wild-type KatG, the spectrum observed immediately after mixing (1.3 ms, Figure 5B, bold line) was followed by transition to a spectrum that is usually observed by mixing PAA with ferric KatG (Figure 5B, black line). The same transition was observed in Arg439Ala (14). Because of the low catalatic activity of Arg439Ala it was possible to monitor compound I formation with hydrogen peroxide. Based on these findings the same approach was used for the variant Lys435Gln, which exhibits also diminished (~50%) catalatic activity. Nevertheless, it was not possible to monitor compound I formation using hydrogen peroxide, most probably because the catalatic activity of Arg439Ala was significantly lower (4.9% of the wild-type acitivity) than that of Lys435Gln (14).
Figure 4. Reaction of 2 µM KatG variant Arg509Lys with 5 mM hydrogen peroxide. Spectra were recorded immediately after mixing (1.3 ms, bold line) and after 470 ms, 700 ms and 10 s (gray line). Conditions: 50 mM phosphate buffer, pH 7.0, and 25°C.

Figure 5. Reaction of KatG variant Lys435Gln with hydrogen peroxide. (A) Reaction intermediates monitored 1.3 ms after mixing of 2 µM Lys435Gln (gray line) with 200 µM H₂O₂ (black line) or 2 mM H₂O₂ (bold line). Conditions: 50 mM phosphate buffer, pH 7.0, and 25°C. (B) Reaction of 2 µM Lys435Gln (gray line) with 200 µM H₂O₂. Bold line depicts the spectrum immediately after mixing (1.3 ms) followed by compound I (black line) formed after 150 ms. Conditions as in (A).

Compound I reduction with ascorbate – Addition of one-electron donors to peroxidase compound I leads to the formation of compound II. A typical plant peroxidase type compound II, as observed in the KatG variant Tyr249Phe (16), exhibits a Soret band red-shifted to 418 nm and two additional peaks at 535 and 560 nm. This is in contrast to wild-type KatG (10) and all KatG variants analyzed within this work, which show different behavior. The time trace at 407 nm, extracted from the spectra, shows that compound I reduction with ascorbate follows a biphasic curve with a fast initial phase followed by a slower phase. The intermediate formed in wild-type KatG after full depletion of ascorbate exhibits a Soret maximum at 407 nm and a distinct peak at 626 nm (10). The spectral transitions in the variant Arg509Lys are shown in Figure 6, with the first spectrum 1.3 ms after mixing, resembling compound I (prepared with PAA) in grey, and
the spectrum of the intermediate after the fast phase (500 ms) with the Soret band at 409 nm and a new peak at 626 nm in black. The last spectrum (Figure 6, bold line), recorded after 10 s, exhibits the same absorption maxima like the 500 ms spectrum but more pronounced.

Figure 6. Reduction of Arg509Lys compound I with ascorbate. The first spectrum was recorded 1.3 ms after addition of ascorbate (gray line) still resembles that of compound I (formed with 50 µM PAA, delay time of 3.5 s). The following spectra were recorded 500 ms (black line) and 10 s (bold line) after mixing of compound I with ascorbate. Conditions: 1 µM enzyme, 2 mM ascorbate, 50 mM phosphate buffer, pH 7.0, and 25°C.

Discussion

Based on structural analyses (6) and sequence alignment a putative peroxidase substrate binding site was investigated by mutational analysis together with kinetic analysis. The U-shaped cleft on the KatG surface comprises some highly conserved residues, from which five were mutated within this work. Exchange of the residues Asn278, Glu281, Lys435, Trp459, and Arg509 did not alter the overall secondary structure of the mutant proteins, which is mainly α-helical. Both Lys435 variants are, besides Arg439Ala, the only recombinant oxidoreductases with decreased catalatic activity compared to wild-type KatG. In Lys435Gln $k_{cat}$ was 49% and in Lys435Arg 70% of wild-type activity. Unfortunately, the peroxidase activity of the variants was even less affected upon mutation. The only variant with reduced oxidation capacity of both substrates was Lys435Arg, although the effect was relatively small. Exchange of Lys435 with glutamine led (similar to Arg439Ala) to a slightly increased peroxidase activity using ABTS in combination with hydrogen peroxide. This increase in the peroxidase activity using ABTS in combination with hydrogen peroxide correlates roughly with the decrease in catalatic activity since both pathways compete for hydrogen peroxide. Interestingly, this effect was not observed upon using guaiacol in combination with hydrogen peroxide (Table 1).
The reduced catalatic activity in Lys435Gln fit with the findings that the spectral features and kinetic transitions upon mixing with hydrogen peroxide are significantly different to wild-type KatG (9). In detail, the first spectrum obtained immediately after mixing with hydrogen peroxide showed a red-shifted Soret band and two additional peaks at 545 and 678 nm followed by transition to a spectrum that is obtained usually by mixing with PAA. The same transitions were observed in the Arg439Ala variant (14) but unlike in this variant compound I formation mediated by hydrogen peroxide could not be monitored in Lys435Gln. In contrast to hydrogen peroxide mediated spectral transitions there was no difference in oxidation kinetics of all seven ferric proteins by PAA.

Compound I reduction with ascorbate in the variants followed the same kinetic and spectral transitions as observed in the wild-type enzyme (10). The reaction starts with a fast phase followed by a slower phase. The spectrum obtained after the initial fast phase did not change until all ascorbate was consumed and exhibits features different from a typical plant type compound II (observed also in KatG variant Trp249Phe), namely a low-spin oxoiron(IV) species. The Soret band in wild-type and mutant KatG compound II is only slightly (1-2 nm) shifted compared to the ferric protein and showed a new band at 626 nm. The spectrum is indicative of a HS ferric form.

To sum up, the region around the “flipping” arginine (Arg439) that forms an U-shaped cleft consists of highly conserved residues. This peculiar cleft on the protein surface combined with the arginine 439 on the surface at the bottom of the “U” was probed as putative one-electron donor binding site that bridges substrate binding and oxidation with the heme cavity. However, neither the catalase nor the peroxidase activity was altered significantly by mutation. Regarding the catalase activity this was as expected since H₂O₂ delivery, reduction and oxidation needs the intactness of the main access channel. And this architecture was apparently not affected in the investigated proteins. However, the only small effects on peroxidase activity, though using one-electron donors of different size and chemistry (guaiacol and ABTS) also suggest that this region is not involved in binding and oxidation of at least small aromatic substrates. Consequently, both the substrate binding site and the nature of the endogenous one-electron donor of both prokaryotic and eukaryotic KatG remain unknown.
References


appendix one

Intracellular catalase/peroxidase from the phytopathogenic fungus Magnaporthe grisea: expression analysis and biochemical characterisation of the recombinant protein

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Phytopathogenic fungi such as the rice blast fungus *Magnaporthe grisea* are unique in having two catalase/peroxidase (*KatG*) paralogues located either intracellularly (*KatG1*) or extracellularly (*KatG2*). The coding genes have recently been shown to derive from a lateral gene transfer from a (proto)bacterial genome followed by gene duplication and diversification. Here we demonstrate that *KatG1* is expressed constitutively in *M. grisea*. It is the first eukaryotic catalase/peroxidase to be expressed heterologously in *Escherichia coli* in high amounts, with high purity and with almost 100% haem occupancy. Recombinant MagKatG1 is an acidic, mainly homodimeric, oxidoreductase with a predominant five-co-ordinated high-spin haem *b*. At 25°C and pH 7.0, the *E*° (standard reduction potential) of the Fe(III)/Fe(II) couple was found to be −186 ± 10 mV. It bound cyanide monophasically with an apparent bimolecular rate constant of (9.0 ± 0.4) × 10^5 M^−1 s^−1 at pH 7.0 and at 25°C and with a *K_a* value of 1.5 μM. Its predominantly catalase activity was characterized by a pH optimum at 6.0 and *k_a* and *K_a* values of 7010 s^−1 and 4.8 mM respectively. In addition, it acts as a versatile peroxidase with a pH optimum in the range 5.0–5.5 using both one-electron [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] and two-electron (Br −, I− or ethanol) donors. Structure–function relationships are discussed with respect to data reported for prokaryotic KatGs, as is the physiological role of MagKatG1. Phylogenetic analysis suggests that (intracellular) MagKatG1 can be regarded as a typical representative for catalase/peroxidase of both phytopathogenic and saprotrophic fungi.

**Key words:** catalase, KatG, *Magnaporthe grisea*, oxidative stress, peroxidase, phytopathogenic fungus.
seem to represent two distinct and abundant fungal KatG groups with different structure–function patterns.

Here, we focus on the expression analysis, recombinant production and biochemical characterization of intracellular catalase/peroxidase of *M. grisea* (MagKatG1). The complete ORF (open reading frame) of katG1 (2217 bp) has been successfully cloned and heterologously expressed, enabling a comprehensive investigation of its biophysical and biochemical properties. The spectral, redox and kinetic data presented are compared with those reported for the corresponding prokaryotic enzymes and are discussed with respect to structure–function relationships and physiological role(s) of eukaryotic catalase/peroxidases.

**MATERIALS AND METHODS**

**Organism**

The ascomycete *M. grisea*, strain MA 829 (Cooke), was obtained from the internal collection of BOKU (the University of Natural Resources and Applied Life Sciences), Vienna, Austria. It was first grown on MPG-agar plates (containing 20 g of malt extract, 1 g of peptone, 20 g of glucose and 20 g of agar in 1 litre) for 4–6 days at room temperature (25 °C). For the isolation of RNA and activity monitoring, it was grown in liquid medium with the same composition (without agar) at 25 °C in a shaking incubator at 130 rev./min for 3 days.

**Sequence-similarity searches: PeroxiBase**

All available sequences of peroxidase genes and proteins were systematically annotated, compared and analysed in PeroxiBase (http://peroxibase.isb-sib.ch/; see [20] for details). All sequence names used in the present paper correspond to the nomenclature of PeroxiBase, where links to the corresponding genes and genomes can also be found. The name and identification number of the intracellular catalase/peroxidase from *M. grisea* are MagKatG1 and 2288 respectively; the corresponding UniProt accession number is A4R5S9.

**Multiple sequence alignment**

Multiple sequence alignment of fungal and bacterial KatG proteins was performed with ClustalX, Version 2.0.5 [21]. The following optimized parameters were applied: Gonnet protein weight matrix with gap opening penalty, 8; gap extension penalty, 0.2; and gap separation distance, 4. The aligned output was presented using GeneDoc, a tool for editing and annotating multiple sequence alignments developed by K.B. Nicholas and H.B. Nicholas in 1997 (http://www.genedoc.us/).

**Promoter and subcellular targeting prediction**

Prediction of intron and native promoter location within the genomic DNA of *M. grisea* strain 70-15 was performed in the SoftBerry suite (http://www.softberry.com) using the ascomycete-specific parameters.

**Cloning, heterologous expression and purification of recombinant MagKatG1**

The cloning strategy for the cDNA and the complete *katG1* gene is described in Supplementary Figure S1 at http://www. BiochemJ.org/bj/418/bj4180443add.htm. Briefly, the complete gene was first maintained in TOPO vector (Invitrogen) and, after modification, it was expressed within the pET21d vector (Novagen), allowing a His$_6$ (hexahistidine)-tag fusion. Heterologous expression was achieved from *Escherichia coli* BL21 DE3 Star cells (Invitrogen) at 16 °C and addition of haemin (10 μM final concn.) concomitantly with isopropl β-D-thiogalactoside (1 mM final concn.) in the cultivation medium. Cells of 16-hold cultures were centrifuged for 15 min at 4500 g, and pellets were resuspended in homogenization buffer containing 50 mM sodium phosphate, pH 8.0, and protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin). Homogenization was performed by two ultrasonication cycles on a Vibra-Cell Model CV17 (Sonics Materials Inc.) sonicator (length of each cycle, 45 s; pulser 50 %; intensive cooling between the cycles). The crude homogenate was centrifuged for 20 min at 20 000 g and the cleared supernatant was applied on to a 30 ml MCAC (metal chelate affinity chromatography) Chelating Sepharose Fast Flow (GE Healthcare) column loaded with Ni$^{2+}$ ions. After loading, the column was washed with 150 ml of buffer A (50 mM sodium phosphate, pH 8.0, containing 500 mM NaCl and protease inhibitors). Bound His-tagged protein was eluted with a linear gradient of buffer A to 100 % buffer B (50 mM sodium phosphate, pH 6.5, containing 500 mM NaCl and 500 mM imidazole). For final purification and determination of putative oligomeric structure of MagKatG1, gel-permeation chromatography (Superdex 200 prep grade; GE Healthcare) was performed. The column volume was 250 ml and the sample volume was 1 ml. The buffer was 5 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl, and was pumped at a flow rate of 0.5 ml/min and a maximal pressure: 0.3 MPa.

The purity of fractions obtained was analysed by SDS/PAGE under standard conditions using a Bio-Rad Mini-Protean system employing a 12 % (w/v) polyacrylamide separating gel. The gels were either stained with Coomassie Brilliant Blue or blotted to nitrocellulose membrane (Amersham Biosciences) for the detection of MagKatG1 by immunoblotting using a polyclonal antibody raised against catalase/peroxidase from *Neurospora crassa* [23].

To reveal the expression pattern of MagKatG1, native PAGE with a linear gradient of 4–18 % acrylamide was used. Native electrophoresis was performed with 10 mM Tris/HisCl buffer, pH 8.3, containing 14.4 g/l glycine in the Mini-Protean apparatus at 150 V for 30 min, followed by 65 V for 3 h. Catalase staining was performed as described in [24], with catalytically active bands occurring as white bands on a dark-green background. Peroxidase activity staining was performed by using o-dianisidine as the electron donor.

**MS analysis**

In order to analyse proteolytic degradation of MagKatG1, bands in the SDS/PAGE gels were excised, destained, carboximethylated and digested with sequencing-grade bovine trypsin (Sigma–Aldrich). The extraction from gel pieces was performed in the same mode as described in [25]. Extracts were dried in a SpeedVac® concentrator and reconstituted with water containing 0.1 % formic acid. Subsequent Matrix-assisted laser desorption ionization–time-of-flight MS analysis was performed on a Voyager-DE STR (Applied Biosystems) instrument with α-cyano-4-hydroxycinnamic acid as matrix.

**ECD (electronic CD) spectrometry and structure prediction**

ECD spectra were recorded on a PIlStar-180 Spectrometer equipped with a thermostatically controlled cell holder (Applied Photophysics). For recording far-UV spectra (260–190 nm), the quartz cuvette had a path length of 1 mm. The spectral bandwidth was 5 nm, the step size was 1 nm, the scan time was 624 s and the
protein (MagKatG1) concentration was 4 μM. Protein concentration was calculated from the known amino acid composition and $A_{280}$, as described in [26]. All ECD measurements were performed in 5 mM phosphate buffer, pH 7.0, at 25°C. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell. The instrument was flushed with nitrogen with a flow rate of 5 litres/min.

In addition, the secondary structure of MagKatG1 was predicted with PSIPRED [27]. The 3D (three-dimensional) structure of MagKatG1 was modelled using EsyPred 3D [28] and KatG from Burkholderia pseudomallei, the bacterium causing melioidosis (1MWV). The reliability of the obtained model was verified using What IF (http://swift.cmbi.ru.nl/servers/htmL/index.htmL).

Electronic UV–visible spectroscopy

The UV–visible spectrum of purified recombinant ferric MagKatG1 in 5 mM phosphate buffer, pH 6.5, was routinely recorded on a Zeiss Specord 10 diode-array photometer over the range 200–800 nm, at a scan speed of 1000 nm/min. The Soret band for ferric MagKatG1 was recorded after mixing MagKatG1 with a flow rate of 5 litres/min.

In order to probe haem accessibility, apparent bimolecular rate constants for the oxidation of dimedone were calculated from the known amino acid composition and $A_{280}$, as described in [26]. All ECD measurements were performed in 5 mM phosphate buffer, pH 7.0, at 25°C. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell. The instrument was flushed with nitrogen with a flow rate of 5 litres/min.

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Spectroelectrochemistry

In order to probe haem accessibility, apparent bimolecular rate constants for the oxidation of dimedone were calculated from the known amino acid composition and $A_{280}$, as described in [26]. All ECD measurements were performed in 5 mM phosphate buffer, pH 7.0, at 25°C. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell. The instrument was flushed with nitrogen with a flow rate of 5 litres/min.

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Overall kinetic parameters

Catalase activity was determined both polarographically using a Clark-type electrode (YSI 5331 oxygen probe) inserted into a stirred water bath (YSI 5301B) or spectrophotometrically by measuring the decomposition of H2O2 at 240 nm as described in [30]. For spectrophotometric measurements, one unit was defined as the amount of enzyme catalysing the conversion of 1 μM of H2O2/min at an initial concentration of 15 mM H2O2 at pH 7.0 and 25°C.

Peroxidase activity was monitored spectrophotometrically using 1 mM H2O2 and 5 mM ABTS ($\varepsilon_{340}$ 31.1 M$^{-1}$·cm$^{-1}$) [31] or 5 mM guaiacol ($\varepsilon_{470}$ 26.6 M$^{-1}$·cm$^{-1}$) or 1 mM o-dianisidine ($\varepsilon_{490}$ 11.3 M$^{-1}$·cm$^{-1}$) or 1 mM pyrogallol ($\varepsilon_{420}$ 2.5 M$^{-1}$·cm$^{-1}$). One unit of peroxidase was defined as the amount of enzyme that decomposes 1 μmol of electron donor/min at pH optimum and at 25°C.

Additionally, peroxidase activity with ethanol as a two-electron donor was determined at 340 nm in a coupled reaction using aldehyde dehydrogenase as described previously [32]. Chlorination and bromination activity of recombinant MagKatG1 was followed by halogenation of monochlorodimedone (100 μM) dissolved in 100 mM phosphate buffer, pH 7.0, containing 10 mM H2O2 and either 10 mM bromide or 100 mM chloride. Rates of halogenation were determined from the initial linear part of the time traces using an absorption coefficient for monochlorodimedone at 290 nm of 19.9 M$^{-1}$·cm$^{-1}$ [33]. One unit was defined as the amount that decomposes 1 μmol of monochlorodimedone/min at pH 7.0 and 25°C. Iodide oxidation was monitored at 353 nm (i.e. the absorbance maximum of tri-iodide ($I_3^−$; $\varepsilon_{353}$ 25.5 M$^{-1}$·cm$^{-1}$)) in 100 mM phosphate buffer, pH 7.0, containing 10 mM H2O2 and 10 mM iodide at pH 7.0 and 25°C [34]. One unit was defined as the amount that produces 1 μmol of I$_3^−$ min$^{-1}$ at pH 7.0 and 25°C.
RESULTS AND DISCUSSION

Statistics from PeroxiBase clearly suggest that, in eukaryotic organisms, KatGs are predominantly spread among the Fungal Kingdom. A recent phylogenetic analysis [2] demonstrated the existence of two distinct types of fungal katG genes. Mainly phytopathogenic fungi (e.g. M. grisea or Gibberella zeae, the latter causing head blight of wheat) contain both paralogues, with katG1 most probably encoding an intracellular, and katG2 an extracellular, catalase/peroxidase.

Analysis and comparison of the whole genome of M. grisea [16] with katG1 and katG2 showed a significant difference in the G + C contents (for katG1, 59.2 versus 51.6 %), suggesting an LGT (lateral gene transfer) of katG genes from bacteria to a fungal progenitor, as has been demonstrated for other fungi [3,6]. Interestingly, the rather high G + C contents of MagkatG1 resembled that of various Burkholderia species (58–68 %), thereby indicating a putative proteobacterial ancestor. In order to answer the question of whether only the katG1 gene or a longer genome portion had been transferred by LGT, we also analysed the putative promoter region of MagkatG1 with GENSCANW (http://genes.mit.edu/GENSCAN.html). A conserved 40-bp-long promoter motif was identified at positions −1838 to −1799 (see PeroxiBase ID = 2288 for further details). Moreover, a typical CGGAGT box was found in positions −657 to −652, similar to a promoter region in the catB gene of Aspergillus oryzae [35]. The G + C contents of this 1838-bp-long region is only 49.73 %, thus even lower than the average value for the entire Magnaporthe genome. This suggests that only the coding region was transferred via LGT from a proteobacterial into the fungal genome.

In order to probe whether expression of MagKatG1 is induced by oxidative stress, cultures of Magnaporthe grisea were grown in MPG in the absence and presence of H2O2 or peroxyacetic acid via LGT from a proteobacterial into the fungal genome. This suggests that only the coding region was transferred thus even lower than the average value for the entire genome. A conserved 40-bp-long promoter region in the catB gene of Aspergillus oryzae [35]. The G + C contents of this 1838-bp-long region is only 49.73 %, thus even lower than the average value for the entire Magnaporthe genome. This suggests that only the coding region was transferred via LGT from a proteobacterial into the fungal genome.

In order to probe whether expression of MagKatG1 is induced by oxidative stress, cultures of Magnaporthe grisea were grown in MPG in the presence of H2O2 or peroxyacetic acid or paraquat (final concentrations 0.5–2 mM) added in the middle-exponential or early-stationary growth phase respectively. RT-PCR analysis of total RNA isolated from filtered mycelia allowed quantification of katG1-specific mRNA under various conditions. The RT-PCR signal intensities obtained from various cDNA samples (Figure 1) clearly demonstrate that katG1 is expressed constitutively. Only with paraquat was the expression level enhanced to 120 % compared with that under standard conditions. This might suggest a role of intracellular MagKatG1 in the continuous degradation of H2O2 deriving from fungal metabolism and is in contrast with MagKatG2, which, owing to the presence of a (predicted) signal sequence, is located extra-cellularly and is produced on demand; that is, its expression is significantly enhanced under oxidative stress conditions that could occur during plant attack [17].

Subsequently MagKatG1 was heterologously expressed and purified. The entire ORF (2217 bp) was cloned in TOPO vector using outer primers and modified by introducing NcoI, AgeI and NotI restriction-endonuclease sites without affecting the coding sequence (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/418/bj4180443add.htm). This allowed cloning in pET21d vector (Novagen) in-frame with a C-terminal His6-tag fusion. Heterologous expression of the recombinant plasmid pMzM1 was performed in E. coli strain BL21 DE3 Star (Invitrogen) at 16 °C and, in the presence of haemin, avoided formation of inclusion bodies and produced recombinant protein with almost 100 % haem occupancy. On average about 30 mg of soluble KatG1 could be obtained by lysis from cell pellets of 1 litre of liquid culture. Protein purification included MCAC followed by gel-permeation chromatography as described in the Materials and methods section.

Analysis of purity by SDS/PAGE revealed the existence of one major band at 85 kDa corresponding to monomeric MagKatG1 (theoretical molar mass of monomer with one haem b and a His6-tag is 83.17 kDa), but additional minor bands were always present at 160, 51, 31 and 27 kDa respectively, irrespective of variations in the purification protocol. This prompted us to probe the protein pattern by immunoblotting using a polyclonal antibody raised against NcKatG1 (PeroxiBase ID = 2181); also known as NcCat2; see Supplementary Figure S5 at http://www.BiochemJ.org/bj/418/bj4180443add.htm for a sequence comparison). Figure 2(A) shows that all the bands at 51, 31, and 27 kDa gave positive signals after staining suggesting proteolytic degradation of MagKatG1. This was proven by tryptic digestion and peptide mass-mapping (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/418/bj4180443add.htm). The molecular masses of the main peptides produced were fully in accordance with those of peptides obtained by in silico trypsin-mediated digestion of MagKatG1. The band at 160 kDa suggested the presence of a covalently linked dimeric form of MagKatG1.

Native electrophoresis of recombinant MagKatG1 revealed positive catalase and peroxidase stains at the position of the dimeric protein, as well as a minor catalase activity at the position of tetrameric MagKatG1 (Figure 2B). In order to analyse the oligomeric structure further, gel-permeation chromatography was performed (Superdex 200 prep grade calibrated with a Gel Filtration Calibration Kit; GE Healthcare). It clearly demonstrated that about 95 % of the protein existed in homodimeric form (180 ± 4 kDa), whereas about 5 % existed as homotetramer (352 ± 8 kDa) (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/418/bj4180443add.htm), thereby reflecting the data obtained by native electrophoresis. The two so far partially analysed fungal peroxidases isolated directly from Penicillium simplicissimum [11] and N. crassa [12], which both contain only one intracellular KatG, were reported to
be homodimeric non-covalently linked proteins. Concomitant occurrence of homodimeric, with some homotrimeric, KatGs has been reported from prokaryotic proteins [7].

The pI of recombinant MagKatG1 was determined to be 5.7 ± 0.2, which is in good agreement with the calculated value of 6.0 using the ExPaSy server (http://www.expasy.org/tools/pi_tool.html).

In order to gain more insight into the structural features of recombinant MagKatG1, both ECD spectroscopy as well as structure modelling was performed. Figure 3(A) depicts the far-UV ECD spectrum of recombinant MagKatG1 in comparison with catalase/peroxidase from *Synechocystis* (SynKatG) at an identical concentration, i.e. A280. Both the prokaryotic and the eukaryotic enzyme showed the typical features of a predominantly α-helical protein (dichroic bands at 222 and 208 nm). However, the α-helical content of the eukaryotic enzyme is more pronounced, which is also underlined by sequence analysis and secondary-structure prediction using PSIPRED [27] (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/418/bj4180443add.htm).

Figure 3(B) presents a 3D-model of monomeric MagKatG1 in overlay with the known structure of KatG from *B. pseudomallei* (1MWV). Probing the homology model with What IF yielded a root-mean-square Z-score for bond lengths and angles of 0.911 and 1.268, respectively, and a root-mean-square deviation in bond distances of 0.0018 nm (0.018 Å). All predicted bonds were in agreement with standard bond lengths. The active site (Figure 3B) comprises the typical conserved proximal (His279-Asp389-Trp330) and distal (Arg108-His112-Trp111) triads found in all so-far-investigated KatGs [1].

Recent mutational and kinetic analysis of bacterial KatGs revealed peculiar structural features essential for the catalatic activity. These include a unique covalent adduct between distal Trp111 and KatG-typical Tyr238 and Met264 (Figure 3B) [1]. Additionally, it has been demonstrated that a distal aspartate residue (Asp120 in MagKatG1) at the entrance of the channel into the haem cavity contributes to maintenance of a defined hydrogen-bonding network necessary for H2O2 oxidation [1]. All these amino acids are strictly conserved in all prokaryotic and eukaryotic KatGs (see Supplementary Figure S5). One obvious difference between prokaryotic and eukaryotic catalase/peroxidases concerns the KatG-specific loop 1 (LL1 in Supplementary Figure S5B) that is known to contribute to the architecture of the main channel and that controls access to the haem cavity [8]. Owing to the insertion of 23–27 amino acids in fungal KatG1s, LL1 is significantly longer.

More details about the nature of the haem and redox properties were obtained by UV–visible spectroscopy and spectroelectrochemical studies. Figure 4 depicts the electronic UV–visible spectra of HS ferric (Figure 4A), HS ferrous (Figure 4B) and LS ferric (Figure 4C) recombinant MagKatG1 in comparison with those of SynKatG. Ferric MagKatG1 exhibits the typical bands of a haem b-containing peroxidase in the visible and near-UV region with a Soret band at 408 nm, Q-bands at 504 nm and 546 nm and a CT1 band (porphyrin-to-metal charge-transfer band) at 635 nm (Figure 4A). This compares with the corresponding SynKatG absorption maxima at 406 nm (Soret), 502 and 542 nm (Q-bands) and 637 nm (CT1) which have been demonstrated to be representative of a five-co-ordinate HS haem co-existing with a small portion of six-co-ordinate HS haem and a
Figure 3 (A) Electronic UV–visible and far-UV ECD spectra of purified recombinant MagKatG1 and (B) a homology model of the 3D structure of MagKatG1 monomer

(A) Electronic UV–visible and far-UV ECD spectrum (inset) of purified recombinant MagKatG1 (continuous line, 4.2 μM) in 5 mM phosphate buffer, pH 7.0. For comparison the corresponding spectra of SynKatG (broken line) recorded under identical conditions are depicted. (B) Homology model of the 3D structure of MagKatG1 monomer revealed by EsyPred [28]. An overlay with the known structure of KatG from B. pseudomallei (PDB code 1MWV) is presented. The Figure was constructed using the Swiss PDB Viewer.

six-coordinate LS haem, as has been demonstrated by resonance Raman spectroscopy of the prokaryotic protein [1]. The purity number (Reinheitszahl in German), i.e. $A_{408}/A_{280}$, of purified recombinant MagKatG1 varied over the range 0.63–0.65 (Figure 3A), which is comparable with values for heterologously expressed prokaryotic KatGs and indicates 100% haem occupancy [36]. From the two eukaryotic KatGs isolated from P. simplicissimum [11] and N. crassa [12], a Soret absorption at 407 nm and 405 nm respectively was reported. This clearly suggests a very similar architecture of the active site of prokaryotic and intracellular eukaryotic KatGs.

On reduction of ferric MagKatG1 with dithionite in a glove box and recording the spectrum in a gas-tight cuvette, the Soret band shifted to $\approx 432$ nm. A good spectrum of ferrous SynKatG under identical conditions had its peak maxima at 438 nm and at 562 nm, with a shoulder at about 590 nm. Both the position and shape of the Soret band indicate the presence of a mixture of dominating ferrous MagKatG1, with some dioxygen adduct (i.e. compound III), which was underlined by analysis of the second derivative spectrum (results not shown). Despite several trials, we did not succeed in obtaining pure ferrous MagKatG1, which might suggest a higher affinity for dioxygen of the eukaryotic ferrous oxidoreductase compared with the prokaryotic enzyme.

Figure 4(C) depicts the LS cyanide complex of ferric MagKatG1 compared with the cyanide complex of SynKatG. Cyanide converts the HS ($S = 5/2$) iron state to the LS ($S = 1/2$) state, thereby shifting the Soret peak of ferric MagKatG1 from 408 to 419 nm with a clear isosbestic point at 416 nm. An additional peak of the cyanide complex of MagKatG1 was at 536 nm, with a shoulder at 572 nm. This compares with the corresponding peaks of LS SynKatG at 421 and 533 nm.

Determination of the $E^{o}$ of the Fe(III)/Fe(II) couple of recombinant MagKatG1 was performed spectroelectrochemically. Figure 5 shows a representative family of spectra of MagKatG1 at various applied potentials. The midpoint reduction potential ($E^{m}$) was determined from the corresponding Nernst plot (inset to Figure 5). At 25°C and pH 7.0, $E^{o}$ was found to be $(-186\pm10)$ mV, which is 40 mV more positive than that of SynKatG ($-226$ mV) [37], but within the range reported for other...
class I peroxidases such as cytochrome c peroxidase (−194 mV) [38] and ascorbate peroxidase (−160 mV) [39]. This suggests conservation of the structure of the haem b moiety as well as of the electrostatic interactions of the iron ion with the proximal histidine residue (His379) and the immediate surrounding polypeptide matrix. This especially concerns the interaction between the proximal histidine residue and conserved aspartate residue (Asp389) that serves to deprotonate the Nα position of the proximal histidine residue, providing a strong axial ligand with an imidazolate character (Figure 3B). In class I peroxidases, despite their different enzymatic properties, this interaction seems to be carefully optimized in strength and geometry.

Finally, we investigated in addition the kinetics of cyanide binding of one- and two-electron oxidation reactions catalysed by recombinant MagKatG1. In haem protein chemistry, cyanide is a useful ligand to probe accessibility and binding to the haem protein. Cyanide binds to haem proteins at low concentrations due to its high affinity for the iron atom [39]. The apparent second-order rate constant (k_on), calculated from the slope of the linear plot of k_off versus cyanide concentration:

\[ k_{\text{on}} = k_{\text{on}} \cdot [\text{HCN}] + k_{\text{eff}} \]  

(Figure 6C)

was \((9.0 \pm 0.4) \times 10^8 \text{M}^{-1} \cdot \text{s}^{-1}\) at pH 7.0 and 25°C. This compares with \(4.8 \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}\) and \(6.9 \times 10^6 \text{M}^{-1} \cdot \text{s}^{-1}\) determined with KatGs from Synechocystis PCC 6803 KatG [36] and Anacystis nidulans [40]. In both the prokaryotic [1] and eukaryotic (results not shown) KatGs, the pH-dependence of \(k_{\text{on}}\) clearly demonstrates that HCN is the liganding species. From the intercept \((1.4 \pm 0.4 \text{ s}^{-1})\) of the linear plot (Figure 6C), the dissociation rate constant \((k_{\text{off}})\) was obtained, allowing the calculation of the dissociation constant \((K_d)\) from the \(k_{\text{off}}/k_{\text{on}}\) ratio, i.e. 1.5 ± 0.4 μM. This compares with a \(K_d\) of 1.4 ± 0.3 μM obtained by thermodynamic binding studies (Figure 6D). This very small \(K_d\) value might indicate a more opened substrate channel compared with that of bacterial KatGs. This is also supported by the high affinity of O₂ for ferrous MagKatG1 that hampered the formation of pure ferrous protein, even when samples were prepared in the glove box.

Prokaryotic catalase/peroxidases have been demonstrated to be bifunctional enzymes with both a catalatic and peroxidatic activity. The pH profile of both activities of MagKatG1 (Figure 7) showed a sharp maximum, with the catalatic activity being highest at pH 5.0 and the peroxidatic activity at pH 5.5 (substrate guaiacol) and pH 5.0 (substrate ABTS). Recombinant MagKatG1 exhibited a specific catalatic activity of \(3430 \pm 280\) units/mg of protein at pH 6.0 and a specific peroxidatic activity for guaiacol of \(2.7 \pm 0.4\) units/mg at pH 5.5 (Table 1 and Supplementary Figure S6 at http://www.BiochemJ.org/bj/418/bj4180443add.htm). Guaiacol peroxidation was still present at pH 3.0 (0.63 unit/mg of protein). The specific activity of ABTS oxidation at pH 5.0 was determined to be \(32.3 \pm 2.1\) units/mg of protein. In addition, the peroxidatic activity towards o-dianisidine and pyrogallol was determined (Table 1 and Supplementary Figure S6) with the hierarchy of electron donors being:

\[
\text{ABTS} > \text{o-dianisidine} > \text{pyrogallol} > \text{guaiacol}
\]

Whether molecules similar to these compounds play a role in metabolism of this rice-blast fungus and might affect expression of MagkatG1 remains a topic for future investigation.

The apparent \(K_m\) and \(k_{\text{on}}\) for H₂O₂ dismutation (i.e. catalatic activity) were determined at the pH optimum, both polarimetrically and spectrophotometrically at H₂O₂ concentrations ranging from 0.5 to 10 mM. The kinetic parameters were determined to be \(4.8 \pm 0.4\) mM \((K_m)\), 7010 ± 230 s⁻¹ \((k_{\text{on}})\), as well...
as \((1.46 \pm 0.06) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) \((k_{\text{cat}}/K_m)\) respectively, the values thus falling within the range reported for prokaryotic enzymes \((k_{\text{cat}} 9000–15 900 \text{ s}^{-1} \text{ and } K_m 3–5 \text{ mM})\) [7]. It is noteworthy that the apparent \(K_m\) values determined for the KatGs isolated directly from \(P. \text{ simplicissimum}\) and \(N. \text{ crassa}\) were determined to be higher, namely 10.8 mM [11] and 13.0 mM [12] respectively.

In addition, we analysed the capacity of MagKatG1 to oxidize ethanol, halides and NADH. Ethanol oxidation was monitored indirectly in a reaction system that continuously provided ethanol, halides and NADH. Ethanol oxidation was monitored higher, namely 10.8 mM [11] and 13.0 mM [12] respectively.

The concentration of MagKatG1 was 0.2 μM and all measurements were performed at 25°C in 100 mM phosphate or citrate buffers in the range between pH 3.0 and 9.0.

Thermostability tests on MagKatG1 were performed over the range 25–65°C by incubating the enzyme at the corresponding temperature for defined time intervals, followed by measuring the catalase and peroxidase activities at 25°C (Table 2). Comparison with equivalent data for bacterial counterparts [7] revealed that MagKatG1 is a mesophilic enzyme that is more thermostable than \(Synechocystis\) KatG or \(B. \text{ pseudomallei}\) KatG [7]. Generally, the catalase activity is more susceptible to temperature-induced inactivation than is the peroxidase activity. Whether the observed (limited) thermostability of the peroxidatic activity may be related to the fact that \(M. \text{ grisea}\) attacks predominantly various cultivars of rice which grow optimally only in a subtropical climate remains to be investigated.

Summing up, phytopathogenic fungi are unique in having two KatG paralogues located either intracellularly (KatG1) or extracellularly (KatG2). The coding genes have been shown to derive from an LGT from a (proteo)bacterial genome, followed by gene duplication and diversification. MagKatG1 is the first eukaryotic catalase/peroxidase to be heterologously expressed in \(E. \text{ coli}\) in high amounts, with high purity and with almost 100% haem occupancy. The acidic, mainly homodimeric, oxidoreductase contains a predominantly five-co-ordinated high-spin haem \(b\) with spectral and redox properties comparable with those of prokaryotic enzymes. Its overwhelming catalase activity is characterized by a \(pH\) optimum at \(pH\) 6.0 and \(k_{\text{cat}}\) and \(K_m\) values of 7010 s\(^{-1}\) and 4.8 mM respectively. The intracellular metalloenzyme is expressed constitutively and might contribute to continuous degradation of \(H_2O_2\) deriving from fungal metabolism in the cytosol. In addition, the bifunctional enzyme acts as a versatile electron donor for recombinant MagKatG1.

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REFERENCES
**SUPPLEMENTARY ONLINE DATA**

**Intracellular catalase/peroxidase from the phytopathogenic rice blast fungus *Magnaporthe grisea*: expression analysis and biochemical characterization of the recombinant protein**

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### Figure S1  Cloning methodology and heterologous expression of *MagkatG1*

(A) Oligonucleotide primers used for cloning and RT–PCR (introduced restriction sites are shown in bold type). (B) Recombinant plasmid used for the heterologous expression of the *MagkatG1* gene (shown in red, ligated into pET21d vector). This plasmid construct was transformed in E. coli BL21 DE3 Star cells (Invitrogen).

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Figure S2  Peptide pattern obtained by MS analysis of putative (proteolytic) degradation products of recombinant MagKatG1 separated by SDS/PAGE

Selected bands at 160 (A), 83 (B), 51 (C), 31 kDa (D) and 27 kDa (E) were digested with trypsin.
Recombinant M. grisea KatG1 was subjected to Ni<sup>2+</sup> MCAC on a column loaded with Superdex 200 prep grade.
Figure S4  Secondary-structure prediction for MagKatG1 using PSIPRED

Abbreviations used: H, helix; E, strand; C, coil; confidence from 0 (=low) to 9 (=high). Colour scheme for essential residues: magenta, distal side of the prosthetic haem group; cyan, proximal side of haem; orange and yellow, distal and proximal residues respectively that are also found in the (non-haem) C-terminal domain.
Figure S5  Selected parts of multiple sequence alignment (ClustalX) of ten bacterial and 33 fungal catalase/peroxidases

(A) Distal haem side, including Arg108, Trp111, His112, Asp120 and Asn121 (see the arrows), the latter being the H-bonding partner of proximal His112 (MagKatG1 numbering).  
(B) Part of large loop 1.  
(C) Continuation of large loop 1 (including Tyr239) and sequence region containing Met264 (part of the KatG-specific covalent adduct) and proximal His279.  
(D) Sequence region containing the H-bonding partner of proximal His279, i.e. Asp389 and Trp330. The colour scheme corresponds to the level of conservation: blue, highest (invariant); green, moderate; yellow, low. In the interests of brevity, the individual enzymes are not identified.
Figure S6   Selected time traces of the reaction between recombinant MagKatG1 (clone M133) and various electron donors

(A) H$_2$O$_2$ degradation monitored at 240 nm; (B) ABTS oxidation (414 nm); (C) guaiacol oxidation (470 nm); (D) o-dianisidine oxidation (460 nm); (E) pyrogallol oxidation (430 nm); and (F) monochlorodimedone oxidation by released hypobromous acid. For detailed reaction conditions, see the Materials and methods section of the main paper.
appendix two

Protein- and solvent-based contributions to the redox thermodynamics of lactoperoxidase and eosinophil peroxidase

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Protein- and solvent-based contributions to the redox thermodynamics of lactoperoxidase and eosinophil peroxidase

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**Keywords:** Lactoperoxidase, eosinophil peroxidase; reduction potential; redox thermodynamics; enthalpy, entropy, heme cavity, channel architecture.

**Abbreviations:** LPO, lactoperoxidase; EPO, eosinophil peroxidase; MPO, myeloperoxidase; TPO, thyroid peroxidase; HRP, horseradish peroxidase; ARP, *Arthromyces ramosus* peroxidase; KatG, catalase-peroxidase; $E^\circ$, standard reduction potential (pH 7, 25°C); SHE, standard hydrogen electrode; $H^\circ_{rc}$, enthalpy change for the reaction center upon reduction of the oxidized protein; $S^\circ_{rc}$, entropy change for the reaction center upon reduction of the oxidized form; RR, resonance Raman; FT-IR, Fourier transform – infrared.
Abstract

Eosinophil peroxidase (EPO) and lactoperoxidase (LPO) are important constituents of the innate immune system of mammals. These heme enzymes belong to the peroxidase-cyclooxygenase superfamily and catalyze the oxidation of thiocyanate, bromide and nitrite to hypothiocyanate, hypobromous acid and nitrogen dioxide that are toxic for invading pathogens. In order to gain a better understanding of the observed differences in substrate specificity and oxidation capacity in relation to heme and protein structure, a comprehensive spectroelectrochemical investigation was performed. The reduction potential ($E^\circ$) of the Fe(III)/Fe(II) couple of EPO and LPO was determined to be -126 mV and -176 mV, respectively (25°C, pH 7.0). Variable temperature experiments show that EPO and LPO feature different reduction thermodynamics. In particular, reduction of ferric EPO is enthalpically and entropically disfavoured, whereas in LPO the entropic term, which selectively stabilizes the oxidized form, prevails on the enthalpic term that favours reduction of Fe(III). The data are discussed with respect to the architecture of the heme cavity and the substrate channel. Comparison with published data for myeloperoxidase demonstrates the effect of heme to protein linkages and heme distortion on the redox chemistry of mammalian peroxidases and in consequence on the enzymatic properties of these physiologically important oxidoreductases.
Introduction

Vertebrate peroxidases constitute a subfamily of the peroxidase-cyclooxygenase superfamily [1]. The main clades of this subfamily are represented by myeloperoxidases (MPOs) and eosinophil peroxidases (EPOs) that are well separated from the lactoperoxidase (LPO) branch. These soluble heme peroxidases are important constituents of the innate immune system and are released when pathogens and parasites invade the organisms [2-4]. Closely related with their role in host defense is their enzymatic activity. They oxidize small anionic molecules, such as halides (chloride and bromide), thiocyanate, and nitrite to the corresponding oxidation products [2-5]. These involve e.g. hypohalous acids or nitrogen dioxide, i.e. (strong) halogenating and nitrating antimicrobial oxidants. Clearly segregated from these three clades and thus distantly related with MPO, EPO, LPO are thyroid peroxidases (TPOs) that are crucial in hormone biosynthesis [6].

These four heme oxidoreductases are found in all mammals including man, and differ in their ability to bind and oxidize their small inorganic substrate molecules [5]. X-ray structures of mammalian peroxidases have been published for human myeloperoxidase [7-9], as well as for caprine lactoperoxidase [10, 11], whereas the three dimensional structures of both EPO and TPO are still unknown.

In both EPO and LPO, as well as in all vertebrate peroxidases, the methyl substituents of pyrrole rings A and C of heme $b$ form two ester bonds with highly conserved distal aspartate and glutamate residues [3, 7-11, 14] (Figures 1B & 1D). The existence of these covalent bonds disturbs the symmetry and planarity of the prosthetic group thereby modulating the spectroscopic and redox properties of the metalloproteins [14, 15]. This is very pronounced with myeloperoxidases that are singular in having an additional sulfonium ion linkage between the heme 2-vinyl group and a conserved methionine [7-9, 14] (Figures 1A & 1C). X-ray structures of both LPO and MPO clearly show that the heme iron in its resting (ferric) high-spin state is positioned out of plane toward the proximal histidine, that is hydrogen bonded to the NH$_2$ group of a fully conserved asparagine [9] (Figure 1D). This proximal heme site architecture is a typical feature for vertebrate peroxidases. Furthermore, the distal site of both LPO and MPO accommodates a conserved distal glutamine residue (besides the peroxidase-typical His-Arg couple) and a complex hydrogen bonding network that includes several conserved water molecules leading from the substrate channel to the heme iron, which supports halide delivery and binding (Figures 1E & 1F). Moreover, an array of conserved water molecules might
Figure 1. Covalent binding pattern of heme in (A) myeloperoxidase (MPO) and (B) in lactoperoxidase (LPO) and eosinophil peroxidase (EPO). In MPO the heme is covalently bonded via two ester bonds to D94 and E242, as well as via a sulfonium ion linkage to M243. In addition the conserved distal residues H95, Q91, Glu242, and a set of aromatic residues (W1, W2, W3, W4, W5, W6) provide additional heme stabilization.
R239 and Q91, as well as proximal H336 and N421 are shown (C). With the exception of the MPO-typical sulfonium ion linkage, the same residues are conserved in LPO (D) and – suggested by sequence alignment [15] in EPO. Figures (E) and (F) depict the conserved water molecules in the distal heme side of MPO and EPO. This panel was constructed using the following coordinates deposited in the Protein Data Bank. MPO: 1CXP; LPO: 2NQX (B) and 3GC1 (F).

facilitate proton release from hydrogen peroxide to the surface of the protein [7, 10, 11, 16].

The H$_2$O$_2$-mediated two-electron oxidation of peroxidases to compound I [oxoferryl species, Fe(IV)=O, plus porphyril or protein radical] requires stabilization of the ferric state that rapidly reacts with H$_2$O$_2$. This is usually guaranteed by the reduction potentials ($E^{\circ}$) of the Fe(III)/Fe(II) couple, which range from -180 to -300 mV (vs. SHE) [14, 17-21]. The $E^{\circ}$ value of the Fe(III)/Fe(II) couple of LPO has been reported to be -170 mV [17], whereas that for MPO is distinctively high (+5 mV) [18], being similar to those of globins, due to the electron-withdrawing sulfonium ion linkage [14, 15]. No redox data about EPO and TPO are available so far.

Here, the standard reduction potential $E^{\circ}$ [(Fe(III)/Fe(II)] of EPO and the changes in enthalpy, $\Delta H^{\circ}_{rc}$, and entropy, $\Delta S^{\circ}_{rc}$, accompanying Fe(III) to Fe(II) reduction in EPO and LPO have been determined for the first time by spectroelectrochemical experiments carried out at different temperatures. These thermodynamic data are compared with those of MPO [18] and some members of the non-animal heme peroxidase superfamily [19-21] and discussed with respect to structural and functional differences between the two superfamilies as well as between the four clades of mammalian peroxidases, obtaining new hints concerning the molecular determinants of $E^{\circ}$ in this important class of metalloenzymes.

**Materials and Methods**

Lactoperoxidase from bovine milk was purchased as a lyophilized powder (Sigma Chemical Co. type L-8257, purity index A$_{412}$/A$_{280}$ > 0.9). Essentially salt-free protein was dissolved in 100 mM phosphate buffer and 100 mM NaCl (pH 7.0), and concentration was determined by using the extinction coefficient 112,000 M$^{-1}$ cm$^{-1}$ at 412 nm [22]. Lyophilized salt-free human eosinophil peroxidase with a purity index (A$_{413}$/A$_{280}$) > 1 was obtained from Planta Natural Products (http://www.planta.at). Its concentration was determined at Soret maximum at 413 nm (110,000 M$^{-1}$ cm$^{-1}$) [23].

All chemicals were reagent grade.
Spectroelectrochemistry. All experiments were carried out in a home-made OTTLE cell [18-21, 24-26]. The three-electrode configuration was realized with a gold minigrid working electrode (Buckbee-Mears, Chicago, IL), a Ag/AgCl/KCl sat microreference electrode, separated from the working solution by a Vycor set, and a platinum wire as the counter electrode. The reference electrode was calibrated against a saturated calomel electrode before and after each set of measurements. All potentials are referenced to the SHE. Potentials were applied across the OTTLE cell with an Amel model 2053 potentiostat/galvanostat. The functioning of the OTTLE cell was checked by measuring the reduction potential of yeast iso-1-cytochrome c in conditions similar to those used in the present work [18-21, 24-26]. The $E^\circ$ value was identical to that determined by cyclic voltammetry (+260 mV) [26]. The temperature of the OTTLE cell, controlled with a circulating water bath, was measured with a Cu-costan microthermocouple. UV-vis spectra were recorded using a Varian Cary C50 spectrophotometer.

Variable temperature experiments were carried out using a “non-isothermal” cell configuration [18-20, 26-29], namely, the temperature of the reference electrode and the counter electrode was kept constant, whereas that of the working electrode was varied. For this experimental configuration $\Delta S^\circ_{rc}$ is calculated from the slope of the $E^\circ$ versus temperature plot, whereas $\Delta H^\circ_{rc}$ is obtained from the Gibbs-Helmholtz equation, namely, from the slope of the plot of $E^\circ/T$ versus $1/T$ [30].

All experiments were carried out under Argon over the 15-35 °C range using 1 mL samples containing 6 µM LPO and 4 µM EPO dissolved in 100 mM phosphate buffer and 100 mM NaCl at pH 7.0. Mediators were used as follows: 36 µM (LPO) or 20 µM (EPO) methyl viologen and 2 µM (LPO) or 1 µM (EPO) lumiflavine-3-acetate, indigo disulfonate, phenazine methosulfate, and methylene blue. Nernst plots consisted of at least five points and were found to be linear, with a slope consistent with a one-electron reduction process.

Results and Discussion

$E^\circ$ [Fe(III)/Fe(II)]. The electronic spectra for bovine lactoperoxidase and human eosinophil peroxidase at different applied potentials in the OTTLE cell (25°C, pH 7) are shown in Figure 2, along with the corresponding Nernst plots (insets to Figure 2). The electronic absorption spectrum of Fe(III) LPO shows a fairly sharp Soret band at 412 nm and maxima in the visible range at 501 nm, 545 nm, 595 nm, and 631 nm, very similar to those reported in the literature [13, 14, 31]. Reduction of ferric LPO proceeds through the
formation of a transient ferrous intermediate with band maxima at 444 nm (Soret band), 561 nm, and 593 nm, which was not stable but converted slowly within 45-60 minutes into a new stable Fe(II) form of LPO, characterized by absorption maxima at 434 nm (Soret band), 561 and 593 nm [31, 32]. This process is due to an equilibrium between a protein form with a relatively open, unrestricted heme pocket, which transforms into a more constrained conformer [31-33]. This phenomenon is typical for LPO, and has been observed neither with MPO [31] nor with EPO (see below). Thus, to avoid any interference from the transient Fe(II) intermediate, in this work LPO was first fully reduced electrochemically, allowing its complete conversion into the stable ferrous form, which was then subjected to (oxidative) spectroelectrochemical titrations. Therefore, the $E^{\circ'}$ values and the corresponding reduction thermodynamics refer to the redox equilibrium between the stable Fe(II) form of LPO and the ferric species.

The Nernst plots obtained from the spectroelectrochemical titrations of LPO are linear (inset to Figure 2A) with a slope close to the theoretical value of $RT/F=0.059$ V at 25 °C, as expected for the one-electron Fe(III)/Fe(II) redox process [18-20, 26-29, 34-36]. The calculated standard reduction potential $[E^{\circ'}(\text{Fe(III)/Fe(II)})$] of bovine LPO of -176 ± 5 mV (25°C and pH 7.0) is slightly higher than a previous literature value (190 mV) [17], probably as a consequence of differences in the ionic composition of the protein solutions that were employed.

Figure 2B shows the electronic spectra of EPO measured at different applied potentials [32]. From the linear Nernst plot (inset to Figure 2B) $E^{\circ'}$ was determined to be -126 ± 5 mV, 50 mV higher than in LPO. This difference is rather unexpected and demonstrates that the similarity between the redox properties of the two peroxidases is less strict than was previously supposed [5, 16].

The $E^{\circ'}$ values for the Fe(III)/Fe(II) couple in LPO and EPO are listed in table 1 along with those for MPO and (non-animal) heme peroxidases with unmodified heme b. The $E^{\circ'}$ values for LPO and EPO were 181 mV and 131 mV lower than in MPO [18], unequivocally underlying the impact of the peculiar electron withdrawing sulfonium ion bond and of the pronounced heme distortion on the redox chemistry of heme iron in myeloperoxidase. Interestingly, the hierarchy of $E^{\circ'}[\text{Fe(III)/Fe(II)}]$, namely MPO > EPO > LPO, follows the same trend that was observed for $E^{\circ}$ of the compound I/Fe(III) couple, i.e. MPO (1160 mV) > EPO (1100 mV) > LPO (1090 mV) [37, 38]. This suggests that within a defined peroxidase the same molecular factors influence the redox properties of the heme iron at different oxidation states. Additionally, it nicely reflects the capacity of
these oxidoreductases to oxidize halides. Only MPO is able to oxidize chloride \([E^{0'} (\text{HOCl, Cl}^-, \text{H}_2\text{O}) = 1280 \text{ mV}]\) at reasonable rates, and bromide oxidation \([E^{0'} (\text{HOBr, Br}^-, \text{H}_2\text{O}) = 1130 \text{ mV}]\) mediated by EPO compound I is faster than by LPO compound I [5, 16].

![Figure 2](image1)

**Figure 2.** Electronic spectra of high-spin bovine LPO (A) and human EPO (B) obtained at various potentials. Spectra were recorded at 25°C. The insets depict the corresponding Nernst plots, were \(X\) represents \((A_{\text{red max}} - A_{\text{ox max}})/(A_{\text{ox max}} - A_{\text{ox}})\). In case of LPO (A) \(\lambda_{\text{ox max}} = 412 \text{ nm and } \lambda_{\text{red max}} = 434 \text{ nm, in case of EPO } \lambda_{\text{ox max}} = 413 \text{ nm and } \lambda_{\text{red max}} = 437 \text{ nm.}\)

Due to the lack of the three dimensional structure of EPO, the observed differences between LPO and EPO cannot be related to distinct structural features. In both proteins, heme \(b\) is covalently bound by two ester bonds [10, 11, 39] resulting in similar spectral properties. Compared with (non-animal, plant-type) peroxidases with unmodified heme \(b\), both LPO and EPO have more positive reduction potentials of the Fe(III)/Fe(II) couple (Table 1). This indicates that the two heme to protein ester bonds affects the redox chemistry of the heme iron, in agreement with theoretical calculations [15]. Apparently these effects are more pronounced in EPO than in LPO.
The electron density at the heme iron in peroxidases is sensibly influenced by the imidazolate character of the proximal histidine, which depends on its H-bonding partner that is a conserved aspartate and a glutamine in heme b (plant-type) peroxidases and in vertebrate peroxidases, respectively. In the former enzymes, it is generally assumed that the Asp-His-iron interaction at the proximal heme side mainly contributes to the (negative) \( E^\circ \) by deprotonating the N\( ^\delta \) position of the proximal histidine. This guarantees the stability of the ferric form that is competent in binding and reducing of hydrogen peroxide. In vertebrate peroxidases, the unprotonated N\( ^\delta \) of the proximal histidine (anionic) is hydrogen bonded with the NH\( _2 \)-group of a fully conserved asparagine (Figure 1 D), whose carbonyl group interacts with the guanidinium group of a conserved arginine [9]. The pronounced imidazolate character of the proximal histidine is confirmed by resonance Raman studies in the low-frequency region [14] that demonstrated that the iron-imidazole stretching mode of both LPO and MPO is at fairly high frequencies. These data suggest that – independent of the nature of the H-bonding partner – in both heme peroxidase superfamilies the proximal histidine has a pronounced imidazolate character, that strengthens the iron-imidazole bond and exerts a comparable influence on the \( E^\circ \) of the heme iron, stabilizing its higher oxidation states.

**Table 1.** Thermodynamic parameters for the Fe(III) \( \rightarrow \) Fe(II) reduction in human eosinophil peroxidase (EPO) and bovine lactoperoxidase (LPO)\(^a\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>( E^\circ ) (V)(^b)</th>
<th>( \Delta H^\circ_{\text{rc}} ) (kJ mol(^{-1}))</th>
<th>( \Delta S^\circ_{\text{rc}} ) (JK(^{-1}) mol(^{-1}))</th>
<th>( \Delta H^\circ_{\text{rc}}(\text{int}) ) (=( \Delta G^\circ = -nF E^\circ )) (kJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO</td>
<td>-0.126</td>
<td>+7</td>
<td>-18</td>
<td>+12</td>
</tr>
<tr>
<td>LPO</td>
<td>-0.176</td>
<td>-5</td>
<td>-73</td>
<td>+17</td>
</tr>
<tr>
<td>MPO [18]</td>
<td>+0.005</td>
<td>+3</td>
<td>+10</td>
<td>0</td>
</tr>
<tr>
<td>HRP [21]</td>
<td>-0.306</td>
<td>+91</td>
<td>+210</td>
<td>+29</td>
</tr>
<tr>
<td>ARP [20]</td>
<td>-0.183</td>
<td>-18</td>
<td>-120</td>
<td>+18</td>
</tr>
<tr>
<td>KatG [19]</td>
<td>-0.226</td>
<td>+17</td>
<td>-18</td>
<td>+22</td>
</tr>
</tbody>
</table>

\(^a\)For comparison purposes, the \( E^\circ \) values of the Fe(III)/Fe(II) couple of human myeloperoxidase (MPO), horseradish peroxidase isoform C (HRP), *Arthromices ramosus* peroxidase (ARP) and *Synechocystis* PC6803 catalase-peroxidase (KatG) are presented (ref. in parenthesis). Average errors for \( E^\circ \), \( \Delta H^\circ_{\text{rc}} \), and \( \Delta S^\circ_{\text{rc}} \) are \( \pm0.005\) V, \( \pm4\) kJ mol\(^{-1}\), and \( \pm8\) JK\(^{-1}\) mol\(^{-1}\), respectively.

\(^b\)At 298.15 K and referred to the SHE. Often the sum (\( -\Delta H^\circ_{\text{rc}}/F + 7298\Delta S^\circ_{\text{rc}}/F \)) does not exactly match \( E^\circ \) because the \( \Delta H^\circ_{\text{rc}} \) and \( \Delta S^\circ_{\text{rc}} \) values are rounded to the closest integer, as a result of experimental error.
Reduction thermodynamics. The temperature profiles of the \( E^{\circ} \) values for EPO and LPO are shown in Figure 3, together with that of MPO [18]. In contrast to MPO, the reduction potentials of both LPO and EPO decrease with increasing temperature. The spectroelectrochemical response deteriorates above 35°C due to protein denaturation and solvent evaporation. The obtained temperature profiles allowed factorization of the enthalpy (\( \Delta H^{\circ}_{rc} \)) and entropy (\( \Delta S^{\circ}_{rc} \)) changes during the reduction reaction, which are listed in Table 1 together with those of MPO [18] and non-animal (plant-type) heme peroxidases [19-21].

EPO and LPO feature different reduction thermodynamics. Reduction entropies are negative in both cases, but reduction enthalpy is positive for EPO and negative for LPO. Hence, in EPO Fe(III) reduction is enthalpically and entropically disfavoured, whereas the negative \( E^{\circ} \) value of LPO results from two opposing thermodynamic contributions: an enthalpic term which favors Fe(III) reduction and a larger entropic term that disfavors it.

As discussed thoroughly elsewhere [40], changes in enthalpy and entropy contain contributions arising from both the reorganization of solvent molecules within the hydration sphere of the proteins and from protein-based factors. Thus the enthalpy (\( \Delta H^{\circ}_{rc} \)) and entropy (\( \Delta S^{\circ}_{rc} \)) changes accompanying Fe(III) to Fe(II) reduction in heme proteins are composed of intrinsic protein contributions (\( \Delta H^{\circ}_{rc, \text{int}} \) and \( \Delta S^{\circ}_{rc, \text{int}} \)) and solvent reorganisation factors (\( \Delta H^{\circ}_{rc, \text{solv}} \) and \( \Delta S^{\circ}_{rc, \text{solv}} \)) [18-21, 27, 40] with

\[
\Delta H^{\circ}_{rc} = \Delta H^{\circ}_{rc, \text{int}} + \Delta H^{\circ}_{rc, \text{solv}} \\
\Delta S^{\circ}_{rc} = \Delta S^{\circ}_{rc, \text{int}} + \Delta S^{\circ}_{rc, \text{solv}}.
\]

\( \Delta H^{\circ}_{rc, \text{int}} \) is mainly controlled by the donor properties of the axial heme ligands, the polarity of the protein environment and the electrostatic interactions between the redox center and polar and charged residues surrounding the heme, whereas \( \Delta S^{\circ}_{rc, \text{int}} \) depends on redox state-dependent differences in protein flexibility [18-21, 27, 40]. The available data for the ferric and ferrous forms of heme proteins indicate that, in general, reduction-induced 3D structural changes are quite small, allowing, as a first approximation, to attribute the measured \( \Delta S^{\circ}_{rc} \) to reduction-induced solvent reorganization effects only, i.e. \( \Delta S^{\circ}_{rc, \text{int}} \approx 0 \) [26, 27]. This approach proved to work with plant-type peroxidases (HRP [20, 21], ARP [20], KatG [19]), as well as with MPO [18]. In the latter case, in the absence of structural information concerning the ferrous enzyme, this hypothesis is based on the small local conformational changes observed upon substrate and/or ligand binding to the ferric protein [7, 8]. Given the strict similarity between the structural effect of bromide, thiocyanate and cyanide binding in MPO and LPO [7, 8, 10, 11], the above approach has
been extended to the interpretation of the reduction thermodynamics of lactoperoxidase and eosinophil peroxidise. Hence, it follows that, to a first approximation, for LPO and EPO \( \Delta S^\circ_{\text{rc}} = \Delta S^\circ_{\text{rc, solv}} = -73 \text{ JK}^{-1}\text{mol}^{-1} \) and \(-18 \text{ JK}^{-1}\text{mol}^{-1} \), respectively. This compares with +10 \text{ JK}^{-1}\text{mol}^{-1} \) for MPO [18].

Since reduction-induced solvent reorganization effects (e.g. changes in the H-bonding network involving the water molecules within the hydration sphere of the protein), have been found to induce compensatory enthalpy and entropy changes [40-44], the corresponding enthalpic contribution can be factorized out from the measured enthalpy change, finally allowing estimation of the protein-based contribution to \( \Delta H^\circ_{\text{rc}} \):

\[
\Delta H^\circ_{\text{rc, int}} = \Delta H^\circ_{\text{rc}} - \Delta H^\circ_{\text{rc, solv}} = \Delta H^\circ_{\text{rc}} - T \cdot \Delta S^\circ_{\text{rc}} = \Delta G^\circ_{\text{rc}} = -nFE^\circ \quad (n = 1)
\]

Hence, to a first approximation, the measured \( E^\circ \) coincides with \( \Delta H^\circ_{\text{rc, int}} \) and would ultimately be determined by the selective enthalpic stabilization of one of the two redox states by first coordination sphere and electrostatic effects. As a consequence \( \Delta H^\circ_{\text{rc, int}} = -nFE^\circ \) corresponds to +17 kJ/mol (LPO), +12 kJ/mol (EPO) and 0 kJ/mol (MPO) (Table 1).
Figure 3. Temperature dependence of the reduction potential (A) and \( E^{\circ}/T \) versus \( 1/T \) plots (B) for bovine LPO (●), human EPO (■), and human MPO (▲) (17). The slope of the plot yields \( \Delta S^{\circ}/F \) and \( -\Delta H^{\circ}/F \) values, respectively. Error bars have the same dimensions of the symbols.

**Reduction enthalpy.** Protein-based factors, such as metal-ligand binding interactions and the electrostatics at the interface between the heme, the protein environment and the solvent, selectively stabilize the ferric form of LPO and EPO much more efficiently than in MPO. In plant-type peroxidases the large enthalpic stabilization of the ferric heme (Table 1) has been attributed to the basic character of the proximal histidine and to the polarity of the distal heme cavity, which contains several water molecules involved in an extended network of hydrogen bonds [19-21]. Both sequence analysis [16] and comparison of the published X-ray structures [7-11] suggest that in mammalian peroxidases the heme cavity architecture is conserved. In fact, the anionic character of the proximal histidine, ensuing
from its interaction with the adjacent asparagine [9], as well as the strength of the iron-imidazolate bond and (Figure 1) [31] seem to be a common feature in this family of peroxidases. Similarly, the interactions of the propionate sidechains on pyrrole rings C & D are conserved [7-11, 16]. Also the catalytic amino acids His, Arg and Gln [16] in the distal heme side are strictly conserved (Figures 1C & 1D) as is an extended H-bonding network formed by distinct water molecules (Figures 1E & F), one of which is hydrogen bonded to the Nε of the distal histidine and lies about midway between the Nε of the latter residue and Fe(III).

Based on these observations, it can be assumed that the contribution of these structural features to $\Delta \Delta H^0_{rc, int} = [\Delta H^0_{rc, int}(MPO) - \Delta H^0_{rc, int}(LPO)] = -17 \text{ kJ/mol}$, and therefore to $\Delta E^0 = [E^0 (MPO) - E^0 (LPO)] = 181 \text{ mV}$ is quite small. Thus, it seems that the lower enthalpic stabilization of ferric MPO and the resulting high $E^0$ [Fe(III)/Fe(II)] is mainly a consequence of the sulfonium linkage connecting the sulfur atom of Met243 with the β-carbon of the vinyl group on pyrrole ring A (Figure 1A) [7, 15, 18]. In fact, the positive charge on the sulfur atom, which does not appear to be involved in any additional electrostatic interactions with the protein [7, 8], would electrostatically stabilize the ferrous form of the heme. On the other hand, its electron withdrawing effect reduces the basicity of the four pyrrole nitrogens, thereby decreasing the electron density at the heme iron and lowering the enthalpic stabilization of the ferric form of the enzyme due to ligand binding effects. The latter effect is enhanced by the pronounced distortions imposed on the porphyrin ring in MPO, which further reduces the contacts with the pyrrole nitrogens with the iron ion [7, 8, 31].

The lower protein-based enthalpic stabilization of the ferric form in EPO compared to LPO, $\Delta \Delta H^0_{rc, int} = [\Delta H^0_{rc, int}(EPO) - \Delta H^0_{rc, int}(LPO)] = -5 \text{ kJ/mol}$ and the corresponding $\Delta E^0 = [E^0 (EPO) - E^0 (LPO)] = 50 \text{ mV}$, is difficult to explain due to the absence of structural data [16]. Sequence [16] and mass spectrometric analysis [39] suggest a similar heme cavity architecture and heme to protein binding mode. On the other hand, differences in the electronic UV-vis spectra of the ferrous and ferric form of both enzymes [32] as well in FT-IR data [31, 44] might reflect some differences in the interaction of the heme iron with the proximal histidine and/or the distal water network.
**Reduction entropy.** Since reduction entropy in LPO and EPO is, to a first approximation, mainly determined by reduction-induced solvent reorganization effects within the hydration sphere of the proteins, the observed differences in $\Delta S^{\circ}_{\text{re}}$ cannot be easily correlated with structural features of the proteins unless significant differences exist in protein-solvent interactions, especially at the heme moiety. Qualitatively, the negative $\Delta S^{\circ}_{\text{re}}$ values obtained for LPO and EPO indicate that reduction of Fe(III) increases the ordering of the solvent molecules in the solvation sphere of both proteins. MPO, on the contrary, features a small and positive reduction entropy [18] that has been ascribed to a rigid hydrogen bond network involving conserved water molecules in the surrounding of the heme, which would support the binding of its small anionic substrate and help to transfer and incorporate the oxyferryl oxygen to fixed Cl$^-$ thereby forming hypochlorous acid [16, 18].

Comparison of the 3D-structures of the iodide and bromide complexes of LPO and MPO [8, 11] suggests that the overall rigidity of the hydrogen bond network involving the water molecules in the surrounding of the heme is a common feature of mammalian peroxidases and not limited to MPO. Therefore, the origin of the difference of the $\Delta S^{\circ}_{\text{re}}$ values between LPO and MPO (and EPO) must be searched outside the heme cavity. In MPO the substrate channel is considerably shallow and has a funnel-like shape [7], whereas in LPO the cylindrical access channel is narrower, longer and more hydrophobic (Figure 4) [10, 11]. By contrast, the substrate channels of EPO and MPO feature an 80% sequence homology [16], suggesting a similar channel architecture in both proteins. Therefore, it appears that the heme distal site in LPO is less accessible and less solvent exposed than in MPO and EPO (Figure 4). These differences are mirrored by the $\Delta S^{\circ}_{\text{re}}$ values reported in Table 1. In MPO and EPO Fe(III) reduction is associated with small entropy changes (although of different sign), whereas in LPO reduction entropy is larger and negative. These observations reflect a general feature in metalloprotein chemistry, namely that the reduction entropy turns from positive to negative values with decreasing the solvent accessibility of the metal center [19-21, 25-28, 30].
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