48 Choinowski, T., Blodig, W., Winterhalter, K. H. and Piontek, K. (1999) J. Mol. Biol. **286**, 809–827
49 Doyle, W. A., Blodig, W., Veitch, N. C., Piontek, K. and Smith, A. T. (1998) Biochemistry **37**, 15097–15105
50 Tsukamoto, K., Itakura, H., Sato, K., Fukuyama, K., Miura, S., Takahashi, S., Izekawa, H. and Hosoya, T. (1999) Biochemistry **38**, 12558–12568
51 Poulos, T. L. and Kraut, J. (1980) J. Biol. Chem. **255**, 8199–8205
52 Baek, H. K. and Van Wart, H. E. (1992) J. Am. Chem. Soc. **114**, 718–725
53 Thanabal, V., De Ropp, J. S. and La Mar, G. N. (1988) J. Am. Chem. Soc. **110**, 3027–3035
54 Miller, M. A., Shaw, A. and Kraut, J. (1994) Nat. Struct. Biol. **1**, 524–531
55 Smith, A. T., Sanders, S. A., Greschik, H., Thorneley, R. N. F., Burke, J. F. and Bray, R. C. (1992) Biochem. Soc. Trans. **20**, 340–345
56 Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J. and Kraut, J. (1993) Biochemistry **32**, 9807–9818
57 Fulop, V., Phizackerley, R. P., Soltis, S. M., Clifton, I. J., Wakatsuki, S., Erman, J., Hadju, J. and Edwards, S. L. (1994) Structure **2**, 201–208
58 Edwards, S. L., Poulos, T. L. and Kraut, J. (1984) J. Biol. Chem. **259**, 12984–12988
59 Rodriguez Lopez, J. N., Smith, A. T. and Thorneley, R. N. (1996) J. Biol. Chem. **271**, 4023–4030
60 Dunford, H. B. (1991) Peroxidases in Chemistry and Biology (Everse, J. and Grisham, M. B., eds), pp. 1–24 CRC Press, Boca Raton
61 Ator, M. A. and Ortiz de Montellano, P. R. (1987) J. Biol. Chem. **262**, 1542–1551
62 Cleland, W. W., Frey, P. A. and Gerlt, J. A. (1998) J. Biol. Chem. **273**, 25529–25532
63 Poulos, T. L. and Kraut, J. (1980) J. Biol. Chem. **255**, 10322–10330

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The role of distal tryptophan in the bifunctional activity of catalase-peroxidases

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Abstract

Catalase-peroxidases are bifunctional peroxidases exhibiting an overwhelming catalase activity and a substantial peroxidase activity. Here we present a kinetic study of the formation and reduction of the key intermediate compound I by probing the role of the conserved tryptophan at the distal haem cavity site. Two wild-type proteins and three mutants of *Synechocystis* catalase-peroxidase (W122A and W122F) and *Escherichia coli* catalase-peroxidase (W105F) have been investigated by steady-state and stopped-flow spectroscopy. W122F and W122A completely lost their catalase activity whereas in W105F the catalase activity was reduced by a factor of about 5000. However, the mutations did not influence both formation of compound I and its reduction by peroxidase substrates. It was demonstrated unequivocally that the rate of compound I reduction by pyrogallol or *o*-dianisidine sometimes even exceeded that of the wild-type enzyme. This study demonstrates that the indole ring of distal Trp in catalase-peroxidases is essential for the two-electron reduction of compound I by hydrogen peroxide but not for compound I formation or for peroxidase reactivity (i.e. the one-electron reduction of compound I).

Introduction

Catalase-peroxidases (KatGs) are components of the oxidative defence system of bacterial [1] and fungal [2,3] cells and function primarily as catalases to remove hydrogen peroxide before it can damage cellular components. On the basis of sequence similarities with fungal cytochrome *c* peroxidase (CCP) and plant ascorbate peroxidases (APXs), catalase-peroxidases have been shown to be members of class I of the superfamily of plant, fungal and bacterial peroxidases [4]. KatGs are homomultimeric proteins with monomers approximately twice the size of those of CCP or APXs. This is ascribed to gene duplication [5]. The crystal structures of CCP and APX have been solved [6,7] and sequence alignments with KatGs suggest that all class I peroxidases have conserved the amino acid triad His, Asp and Trp in the proximal pocket and the triad Trp, Arg and His in the distal pocket. Despite this homology, class I peroxidases dramatically differ in their reactivities

Key words: catalase activity, *Escherichia coli*, *Synechocystis* PCC 6803.
Abbreviations used: KatG, catalase-peroxidase; HPI, hydroperoxidase I; SynKatG, catalase-peroxidase from *Synechocystis* PCC 6803; CCP, cytochrome *c* peroxidase; APX, ascorbate peroxidase.
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towards hydrogen peroxide and one-electron donors. Catalase-peroxidases have a predominant catalatic activity but differ from monofunctional catalases in exhibiting also a substantial peroxidatic reaction. On the other hand, no catalase activity was ever reported for both CCP and APX.

Recently, residues in the putative distal active site have been the targets of site-directed mutagenesis studies. The role of distal Trp, Arg and His was studied in hydroperoxidase I (HPI) from *Escherichia coli* [8] and in the catalase-peroxidase from the cyanobacterium *Synechocystis* PCC 6803 (SynKatG) [9]. The data presented in these papers suggested that all three residues have a role in the catalase activity of KatGs. The initial event in the catalytic mechanism is oxidation of the enzyme to an intermediate called compound I. Hydrogen peroxide is the oxidant and, similar to the Poulos–Kraut mechanism [10] proposed for other peroxidases, His and Arg are important in this two-electron oxidation of ferric KatG. Compound I is involved in both the catalase cycle and the peroxidase cycle. In the catalase cycle, a second H_2O_2 molecule is used as a reducing agent of compound I. In both papers [8,9] it was demonstrated that, in this two-electron reduction step of the enzyme, the indole ring of distal Trp is essential. The reasoning for this was based on the observations that in the Trp mutants (i) the catalase activity was significantly reduced [8] or even lost [9], whereas (ii) the peroxidatic-to-catalatic ratio was increased dramatically [8], indicating that compound I formation was not influenced by this mutation.

In order to further characterize the role of distal Trp in the individual reaction steps of a catalase-peroxidase a comparative sequential-mixing stopped-flow study was performed. Wild-type KatGs and Trp mutants from both *E. coli* and *Synechocystis* were investigated in their reaction with H_2O_2 and peroxyacetic acid as well as in compound I reduction by aromatic one-electron donors. It is demonstrated that the phenolic substrates were oxidized by compound I of the Trp mutants at rates that sometimes even exceeded that of the wild-type enzyme, whereas its reactivity towards hydrogen peroxide was decreased dramatically.

Materials and methods

Standard chemicals and biochemicals were obtained from Sigma Chemical Co. Mutagenesis, expression, purification of proteins and spectrophotometric characterization of wild-type and mutant proteins were described previously [8,9].

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) at 25 °C. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2/min at pH 7 and 25 °C. Peroxidase activity was monitored spectrophotometrically using 1 mM H_2O_2 and either 1 mM *o*-dianisidine ($\varepsilon_{460} = 11.3\ mM^{-1} \cdot cm^{-1}$) or 20 mM pyrogallol ($\varepsilon_{430} = 2.47\ mM^{-1} \cdot cm^{-1}$). One unit of peroxidase is defined as the amount that decomposes 1 μmol of electron donor/min at pH 7 and 25 °C. The average of at least three determinations is presented.

Transient-state measurements were made using the model SX-18MV stopped-flow spectrophotometer from Applied Photophysics equipped with a 1 cm observation cell thermostated at 15 °C. Calculation of pseudo-first-order rate constants (k_{obs}) from experimental traces at the Soret maximum was performed with the SpectraKinetic workstation v4.38 interfaced to the instrument. The substrate concentrations were at least ten 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 pseudo-first-order rate constants versus substrate concentration. To follow spectral transitions a photodiode array accessory (model PD.1 from Applied Photophysics) connected to the stopped-flow machine together with the XScan Diode Array Scanning v1.07 software was utilized. For determination of oxidation of ferric catalase-peroxidase to compound I by peroxides the single mixing mode was used. Catalase-peroxidase and peroxyacetic acid or hydrogen peroxide were mixed to give a final concentration of 1 μM enzyme and 20–500 μM peroxide. The first data point was recorded 1.5 ms after mixing and 4000 data points were accumulated. Sequential-mixing stopped-flow analysis was used to measure compound I reduction by one-electron donors. In the first step enzyme was mixed with peroxyacetic acid and, after a delay time where compound I was built, the intermediate was mixed with the electron donor. All stopped-flow determinations were measured in 50 mM phosphate buffer, pH 7.0, and at least three determinations were performed for each substrate concentration.

Results and discussion

Recombinant wild-type KatGs exhibit overwhelming catalase activity. This is underlined by the following data. The polarographically mea-

Table 1

Apparent K_m and k_{cat} for the catalase activity of wild-type catalase-peroxidases and mutants (50 mM phosphate buffer, pH 7, 25 °C)

Also given are the bimolecular rate constants, k_{app}, for the reaction of the ferric enzymes with hydrogen peroxide or peroxyacetic acid forming compound I (pH 7, 15 °C). WT, wild type; PA, peroxyacetic acid; ND, not detectable.

	Catalase activity		Compound I formation $k_{app}(M^{-1} \cdot s^{-1})$	
	K_m (mM H_2O_2)	k_{cat} (s^{-1})	H_2O_2	PA
WT (HPI)	5.9	5300	ND	1.0×10^4
WT (SynKatG)	4.9	3500	ND	3.9×10^4
W105F mutant (HPI)	91	1.1	5.8×10^5	1.1×10^5
W122A mutant (SynKatG)	ND	ND	8.8×10^4	2.1×10^5
W122F mutant (SynKatG)	ND	ND	8.4×10^4	1.8×10^5

Table 2

Specific peroxidase activity (units/mg of protein) of wild-type catalase-peroxidases and mutants (50 mM phosphate buffer, pH 7, 25 °C)

Also given are the bimolecular rate constants, k_{app}, of compound I reduction by pyrogallol and *o*-dianisidine (pH 7, 15 °C). WT, wild type; ND, not determined.

Protein	Pyrogallol (units/mg)	*o*-Dianisidine (units/mg)	Compound I reduction k_{app} ($M^{-1} \cdot s^{-1}$) Pyrogallol	*o*-Dianisidine
WT (HPI)	ND	3.2	1.5×10^5	1.0×10^4
WT (SynKatG)	3.3	2.4	5.4×10^5	3.9×10^4
W105F mutant (HPI)	ND	4.7	2.3×10^5	1.1×10^5
W122A mutant (SynKatG)	2.8	3.2	5.9×10^5	2.1×10^5
W122F mutant (SynKatG)	7.3	2.9	1.9×10^5	1.8×10^5

sured specific catalase activity of recombinant SynKatG used in these studies was 600 ± 26 units/mg of protein in the presence of 1 mM hydrogen peroxide. With 1 mM H_2O_2 and either 5 mM *o*-dianisidine or 20 mM pyrogallol, the peroxidase activity was determined to be 2.4 ± 0.3 units/mg and 3.3 ± 0.5 units/mg, respectively. HPI showed a comparable bifunctional reactivity. The data are summarized in Tables 1 and 2.

We have recently reported that even in a stopped-flow machine it is impossible to see spectral changes when wild-type SynKatG is mixed with hydrogen peroxide [9,11]. This is also the case with wild-type HPI and is most probably related to the high catalase activity and the very fast cycling of compound I back to the resting state of the enzyme (see Figure 2A below). In contrast, with peroxyacetic acid a compound I spectrum can be obtained that is distinguished from the resting state by a 40 % hypochromicity and thus is similar to classical plant peroxidase (e.g. APX) compounds I, which have been shown to contain a porphyrin π-cation radical in combination with an iron(IV) centre [12,13]. This is also supported by recent EPR measurements with HPI [8] and catalase-peroxidase from *Mycobacterium tuberculosis* [14]. Thus, with peroxyacetic acid compound I formation can be followed as an absorbance decrease at the Soret maximum. Plots of k_{obs} values against the peroxyacetic acid concentration yielded apparent bimolecular rate constants of 1.0×10^4 $M^{-1} \cdot s^{-1}$ (HPI) and 3.9×10^4 $M^{-1} \cdot s^{-1}$ (SynKatG). In both cases an at least 50-fold excess of peroxide was necessary for complete formation of compound I, which was stable for at least 20 s.

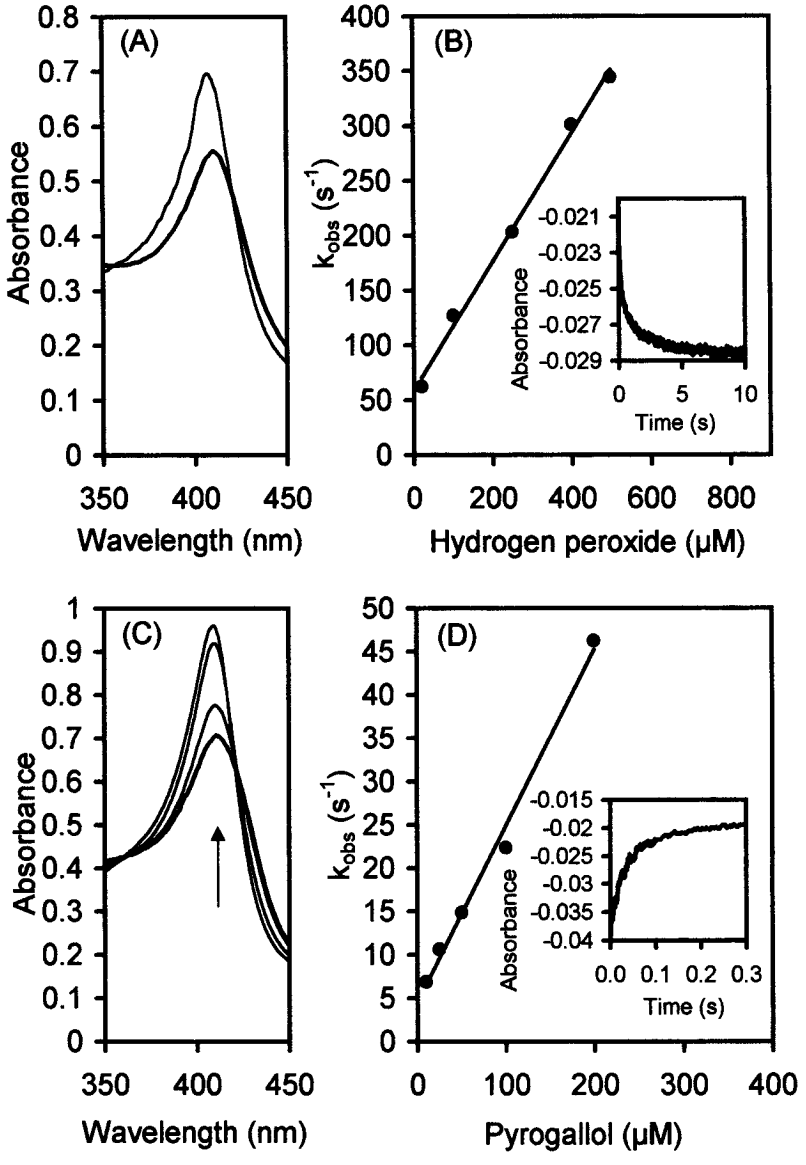
Finally, we have performed sequential-mixing stopped-flow experiments in order to investigate compound I reactivity towards classical

one-electron donors, namely pyrogallol and *o*-dianisidine. In a typical experiment, 4 μM SynKatG was premixed in the aging loop with 200 μM peroxyacetic acid and after a delay time of 20 s the electron donor was added. With HPI the optimum condition was a mix of 4 μM enzyme with 1200 μM peroxyacetic acid and a delay time of 1 s. In each case, a rapid disappearance of compound I and an absorbance increase at the Soret maximum was observed without a spectroscopically distinct (red-shifted) intermediate (compound II) known from plant peroxidases

Figure 1

Absorption spectra and stopped-flow measurements of the mutant W105F of *E. coli* catalase-peroxidase

(**A**) Spectra of ferric enzyme (thin line) and compound I (thick line) formed by mixing 10 μM enzyme with 100 μM hydrogen peroxide and waiting for 5 s. (**B**) Plot of pseudo-first-order rate constants (k_{obs}) for the reaction between W105F and hydrogen peroxide. The inset shows a typical time trace of the reaction of 1 μM enzyme with 100 μM hydrogen peroxide recorded at 407 nm and 15 °C in 50 mM phosphate buffer, pH 7.0. (**C**) Spectral changes upon addition of 2 mM ascorbate to 10 μM compound I. The first spectrum was taken at 1.3 ms. Subsequent spectra were at 20, 700 and 8000 ms. (**D**) Plot of pseudo-first-order rate constants between compound I and pyrogallol. The inset shows a typical time trace of the reaction of compound I with 100 μM pyrogallol recorded at 407 nm and 15 °C in 50 mM phosphate buffer, pH 7.0.

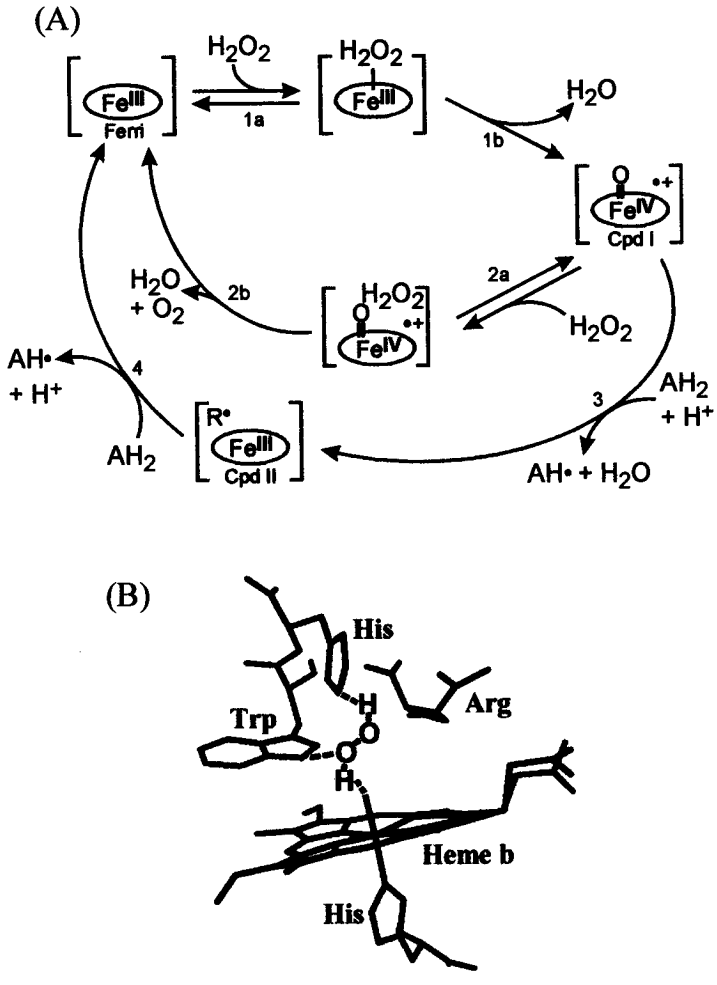


(horseradish peroxidase or APX). Since one-electron donors were added to compound I it is reasonable to assume that compound II is formed, which is either very rapidly reduced further to ferric enzyme and/or has spectral features similar to the native protein. Since a compound II-like species would still be one oxidizing equivalent above the resting state of the enzyme, in the latter case compound I reduction should lead to a protein radical species, as was proposed recently [9,11]. Consequently, we had to follow the direct reaction between compound I and the peroxidase substrates at the Soret maximum of the native enzyme. The time traces exhibited a single-exponential behaviour and the k_{obs} values exhibited a linear dependence on substrate concentration. Pyrogallol was shown to be a $\approx$10-times better electron donor than *o*-dianisidine (Table 2). The rate of

Figure 2

(A) Putative reaction scheme for catalase-peroxidases and (B) arrangement of important amino acids in the active site based on the structure of yeast CCP

(**A**) Putative reaction scheme. In the first step H_2O_2 is used for compound I formation. Compound I (Cpd I) is two oxidizing equivalents above that of the native enzyme with a porphyrin π-cation radical in combination with an iron(IV) centre (reactions 1a and 1b). Compound I can react with a second H_2O_2, reducing the enzyme back to the ferric state (catalase reaction; reactions 2a and 2b). In the peroxidase reaction compound I is transformed in a one-electron reduction to compound II (Cpd II) containing an amino acid radical (R$^{\bullet}$) and Fe(III) (reaction 3). Finally compound II is reduced back to the ferric catalase-peroxidase in a second one-electron reduction (reaction 4). (**B**) Arrangement of important amino acids in the active site of catalase-peroxidases based on the structure of yeast CCP (PDB code 2CYP). The model shows how the distal Trp could be involved in positioning of the second H_2O_2 within the reaction centre of compound I. Hydrogen bonds are shown as broken lines. The figure was constructed using Swiss PDBViewer [17].



compound I reduction was 1.5×10^5 $M^{-1} \cdot s^{-1}$ (HPI) and 5.4×10^5 $M^{-1} \cdot s^{-1}$ (SynKatG) at 15 °C and pH 7.

The Trp variants from both *E. coli* KatG (W105F) and SynKatG (W122A and W122F) differ dramatically from the wild-type proteins in having increased peroxidatic activities (Table 2) and significantly reduced catalatic activity (Table 1). Interestingly, both SynKatG mutants (W122A and W122F) lost their catalase activity, whereas the catalase activity of the HPI mutant (W105F) was decreased by a factor of 5000 compared with wild-type HPI. On the other hand, in all Trp variants the specific peroxidase activity exceeded that of the wild-type protein (Table 2).

These findings fit well with the observation that mutation of distal tryptophan allowed compound I formation by hydrogen peroxide, indicating that these mutations strongly influenced compound I reduction by hydrogen peroxide, but not compound I formation. The inset of Figure 1(B) shows a typical time trace of the reaction of HPI W105 with hydrogen peroxide. Similar kinetics were obtained with peroxyacetic acid and the spectral features of the resulting intermediate compound I were independent of the added peroxide and identical to those obtained with peroxyacetic acid (Figure 1A). With hydrogen peroxide, compound I formation varied between 8.4×10^4 $M^{-1} \cdot s^{-1}$ (SynKatG W122) and 5.8×10^5 $M^{-1} \cdot s^{-1}$ (HPI W105F) at 15 °C and pH 7. For W105F a plot of k_{obs} versus hydrogen peroxide concentration is shown in Figure 1(B). The obtained bimolecular rate constants are about 1–2 orders of magnitude lower than the rates of typical (wild-type) peroxidases [15], suggesting that the exchange of distal tryptophan had some (small) effect on hydrogen peroxide reduction (i.e. compound I formation). With peroxyacetic acid, formation of compound I was even faster than with the wild-type proteins.

In contrast to the catalase activity and consistent with the increase of the specific peroxidase activity, we could demonstrate by the sequential-mixing stopped-flow analysis that compound I reduction by pyrogallol or *o*-dianisidine was not influenced by the replacement of Trp in both catalase-peroxidases. Moreover, the rates of reduction exceeded that of the wild-type enzyme indicating that in all three mutants compound I was peroxidatically hyperactive. Figure 1(C) shows the spectral changes upon mixing ferric W105F with pyrogallol. Similar to wild-type KatGs, re-formation of a ferric-like spectrum is obtained. The inset of Figure 1(D) shows an example of a time trace followed at the Soret maximum of the ferric enzyme which was fitted to first-order kinetics. Since both substrates could contribute in this absorbance region only data for the first 300 ms were fitted and the resulting k_{obs} values plotted against the substrate concentration. In all Trp variants *o*-dianisidine was an 8–10-fold better electron donor compared with the wild-type proteins, whereas with pyrogallol the difference was less pronounced. It is interesting to note that with CCP mutation of distal Trp also led to hyperactive mutants and enhanced oxidation of a number of substituted anilines [16].

Figure 2 proposes a reaction scheme for both the catalase and peroxidase activities of catalase-peroxidases. The first common stage is compound I formation (Figure 2, reaction 1). We could show that Trp is not involved in this two-electron oxidation step. It has been demonstrated recently [8,9] that, similar to other peroxidases, distal histidine and arginine are involved in this step (i.e. the heterolytic cleavage of hydrogen peroxide). The second step of the catalatic process is compound I reduction (Figure 2, reaction 2) back to the resting enzyme, utilizing a second hydrogen peroxide molecule. In this process distal Trp is essential and in Figure 2(B) it is proposed that the indole ring could be involved in hydrogen bonding of the second hydrogen peroxide molecule. But distal Trp is not involved in the two one-electron reduction steps (Figure 2, reactions 3 and 4) which could reflect different locations or orientations of the sites of interaction of the protein with peroxides and aromatic electron donors in catalase-peroxidases.

There are still several questions to be answered. We have shown the importance of the indole ring in the catalase reaction of KatGs. CCP and APXs also have this conserved Trp at the active distal site but do not exhibit catalase activity. Further studies are needed, above all the evaluation of the three-dimensional structure of KatGs, which should help to answer these questions.

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References

1 Loewen, P. C. (1997) in Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J. G., ed.), pp. 273–308, Cold Spring Harbor Press, Cold Spring Harbor
2 Fraaje, M. W., Roubroeks, H. P., Hagen, W. R. and Van Berkel, W. J. H. (1996) Eur. J. Biochem. **235**, 192–198

3 Levy, E., Eyal, Z. and Hochman, A. (1992) Arch. Biochem. Biophys. **296**, 321–327
4 Welinder, K. G. (1992) Curr. Opin. Struct. Biol. **2**, 388–393
5 Welinder, K. G. (1991) Biochim. Biophys. Acta **1080**, 215–220
6 Finzel, B. C., Poulos, T. L. and Kraut, J. (1984) J. Biol. Chem. **259**, 13027–13036
7 Patterson, W. R. and Poulos, T. L. (1995) Biochemistry **34**, 4331–4341
8 Hillar, A., Peters, B., Pauls, R., Loboda, A., Zhang, H., Mauk, A. G. and Loewen, P. C. (2000) Biochemistry **39**, 5868–5875
9 Regelsberger, G., Jakopitsch, C., Rüker, F., Krois, D., Peschek, G. A. and Obinger, C. (2000) J. Biol. Chem. **275**, 22854–22861
10 Poulos, T. L. and Kraut, J. (1980) J. Biol. Chem. **255**, 8199–8205
11 Regelsberger, G., Jakopitsch, C., Engleder, M., Rüker, F., Peschek, G. A. and Obinger, C. (1999) Biochemistry **38**, 10480–10488
12 Patterson, W. R., Poulos, T. L. and Goodin, D. B. (1995) Biochemistry **34**, 4342–4345
13 Marquez, L. A., Quitoriano, M., Zilinskas, B. A. and Dunford, H. B. (1996) FEBS Lett. **389**, 153–156
14 Chouchane, S., Lippai, I. and Magliozzo, R. S. (2000) Biochemistry **39**, 9975–9983
15 Dunford, H. B. (1999) Heme Peroxidases, Wiley-VCH, New York
16 Roe, J. A. and Goodin, D. B. (1993) J. Biol. Chem. **268**, 20037–20045
17 Guex, N. and Peitsch, M. C. (1997) Electrophoresis **18**, 2714–2723

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Engineering the active site of ascorbate peroxidase

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Abstract

Understanding the catalytic versatility of haem enzymes, and in particular the relationships that exist between different classes of haem-containing proteins and the mechanisms by which the apo-protein structure controls chemical reactivity, presents a major experimental and theoretical challenge. These issues are discussed in the general context of peroxidase and cytochrome P450 chemistry, and specific issues relating to the catalytic chemistry of ascorbate peroxidase are highlighted.

Introduction

Iron protoporphyrin IX (haem) is a particularly versatile biological molecule that is capable of an astonishingly diverse range of reactivities [1,2]. As such, it is of continued interest to establish the relationships that exist between different classes of haem-containing proteins, and to rationalize the mechanisms by which the apo-protein structure confers specific chemical reactivities on the haem iron. Such fundamental information is of more than academic interest: it is central to a detailed understanding of the way enzymes operate and should provide a solid conceptual platform for the rational, *de novo* design of new catalytic enzymes. Currently, one of the most effective ways of addressing questions of this kind is using recombinant DNA technology together with high-resolution crystal structure data: systematic perturbations of the protein architecture are introduced and subsequent characterization of the new variant proteins provides useful insight into the functional consequences of the mutation.

One of the most intriguing aspects of haem biochemistry and function that has yet to be fully resolved is the relationship between the peroxidase and cytochrome P450 classes of enzymes [3,4]. Hence, although P450s are thought to utilize as part of their catalytic cycle a transient high-oxidation-state intermediate [5,6] similar to that known to be utilized in peroxidase enzymes [7], they are able not only to catalyse the insertion of 1 mol of oxygen into substrate, a reaction which peroxidases, with a few exceptions (see below), are unable to support, but also to achieve this in an enantioselective manner. These differences in reactivity are poorly understood, but are likely to derive, at least partially, from variations in the axial ligation to the haem and the access of the substrate to the oxidized ferryl species [1,2].

Ascorbate peroxidase (APX)

We have been interested in examining these complex relationships by systematic study of catalytic oxidations in APX [8]. APX activity was first reported in 1979 [9,10] and APX is, therefore, a relative newcomer to the peroxidase field; horseradish and cytochrome *c* peroxidases were, for example, first identified in 1903 [11] and 1940 [12]

Key words: haem, substrate.
Abbreviations used: APX, ascorbate peroxidase; rAPX, recombinant pea cytosolic APX; PC, *p*-cresol.
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