Examining Reactivity and Specificity of Cytochrome *c* Peroxidase by using Combinatorial Mutagenesis

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Combinatorial mutagenesis was used to investigate the role of three key residues in cytochrome c peroxidase (CCP) from Saccharomyces cerevisiae, *Arg48, Trp51, and Trp191, in control of the reactivity and selectivity of the heme-containing enzyme. Libraries were prepared by randomization of these residues and were subsequently screened for activity against the phenolic substrate guaiacol. Screening conditions were employed that favor either mutants with high activity or those with both high activity and stability of the reactive enzyme intermediates. The results obtained suggest a dual role for Arg48 of CCP: in addition to stabilizing reactive enzyme intermediates, the distal arginine residue plays a major role in restriction of access to the ferryl oxygen atom by small molecules and thereby controls reactivity and substrate specificity of the peroxidase. At position 51 of CCP, either a phenylalanine or a tryptophan residue is required both for catalytic and structural reasons. In contrast, either polar or positively charged residues are accepted at the position of Trp191, which is located inside the core of the protein. The variability at position 191 can be interpreted as a reflection of the mechanism of cytochrome c peroxidase, which transforms the nonpolar Trp191 into a transient cation radical.*

KEYWORDS:

directed evolution · enzymes · heme proteins · mutagenesis · protein engineering

Introduction

Peroxidases and other heme-containing enzymes display vast differences in reactivity and substrate specificity. Striking examples for the functional diversity of heme-containing peroxidases can be found within the superfamily of bacterial, plant, and fungal peroxidases, whose members not only oxidize a wide variety of different substrates, but also possess different locations of their substrate binding sites.[1–5] The two most prominent members of this superfamily are cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* and horseradish peroxidase isozyme *c* (HRP).[6, 7] The general mechanism of protoporphyrin-IX-containing peroxidases can be divided into three steps.[6, 7] First, the peroxidase reacts with H_2O_2 to yield one equivalent of water and an oxyferryl ($Fe^{IV}=O$) cation radical called compound I.[3] The cation radical in compound I is either a porphyrin π cation radical or, in the case of CCP, an indolyl cation radical located at Trp191.[8] Two substrate molecules are then oxidized in subsequent one-electron transfer reactions to yield the enzyme back in its Fe^{III} resting state. The intermediate obtained after the first substrate oxidation, an oxyferryl species that results from the reduction of the cation radical, is called compound II. Both steric and chemical properties of the substrate appear to be important for the location of the substrate binding site of a given peroxidase. Whereas the interaction between CCP and its substrate, cytochrome *c*, is mediated for steric reasons by surface residues of the two proteins, HRP binds its phenolic substrates in the distal cavity above the plane of the heme moiety.[9, 10] The location of the binding site of HRP can be rationalized by consideration of the fact that the oxidation of phenols by peroxidases requires simultaneous electron and proton transfer from the phenol to the activated peroxidase.[7, 10] The acceptor for the proton is, in the case of compound I, the distal base or, in the case of compound II, the ferryl oxygen atom.[7, 10] It is the reduction of compound II that is the rate-determining step for the oxidation of phenols.[7]

A variety of factors can be expected to contribute to the 10^3-fold difference in activity of CCP and HRP towards phenols including guaiacol, including specific substrate – enzyme interactions that control substrate binding.[10, 11] One of the fundamental differences in mechanism of the two peroxidases is the nature of the cation radical in compound I. Trp191 harbors the indolyl cation radical in compound I of CCP (Figure 1), whereas the corresponding amino acid in HRP is Phe220. The cation radical in compound I of HRP is thus a porphyrin π cation radical.[8]

Another important difference between CCP and HRP is that HRP possesses a phenylalanine residue (Phe41) at the position

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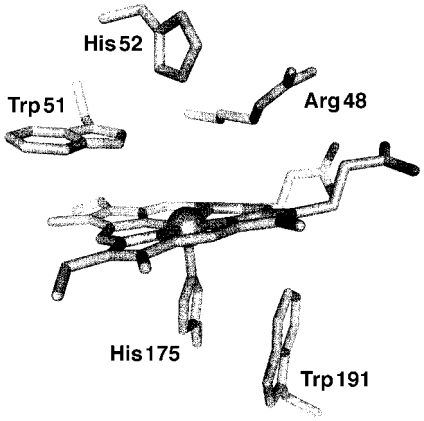

Figure 1. *The locations of Arg48, Trp51, His52, and Trp191 relative to the heme cofactor in the crystal structure of CCP.*[47]

equivalent to that of Trp51 in CCP (Figure 1). Trp51 in CCP forms a hydrogen bond to the ferryl oxygen atom of compound I and compound II.[12, 13] It has been reported that the mutation Trp51Phe increases the reactivity of CCP against cytochrome *c*, anilines, and phenols.[14–16] A possible explanation for this increase in activity is that the removal of the hydrogen bond between Trp51 and the ferryl oxygen atom destabilizes compound I and compound II and thereby increases the reactivity.[14–16] We have recently used directed evolution to generate CCP mutants with increased activity against guaiacol, a typical substrate of HRP.[17] In these experiments, the mutation Arg48His increased the activity against guaiacol about 70-fold. This result was surprising as this arginine residue, which is located in the distal cavity of the peroxidase, is one of only nine residues that are conserved in all members of the superfamily, including those whose natural substrates are phenols (Figure 1).[1]

In general, it is believed that the distal arginine residue of peroxidases aids in the formation and stabilization of compound I.[18–20] In addition, the distal arginine residue (Arg38) in HRP has been shown to play an important role in binding of the substrate during phenol oxidation as hydrogen bonds between the guanidinium group of Arg38 and ferulic acid ideally position the substrate for efficient electron and proton transfer.[10] In HRP and pea cytosolic peroxidase, mutations of the distal arginine residues have also been shown to affect activities that require access to the ferryl oxygen atom.[21–23] To explain the increase in activity against guaiacol for the Arg48His mutants of CCP, we have reasoned that this mutation of CCP increases the steric access of the phenol to both the distal base and the ferryl oxygen atom and thus facilitates the oxidation of the phenol.[17] However, an alternative explanation for the selection of the mutation Arg48His in the directed evolution experiments is that this residue might play an active role in the proton transfer and thus act as a general-acid/base catalyst. It has been shown that the mutation Arg38His in HRP can partially compensate for mutations that remove the distal histidine residue, which functions as the general-acid/base catalyst in the mechanism of action of peroxidases.[21]

In order to investigate the influence of Arg48, Trp51, and Trp191 of CCP on the reactivity and specificity of the peroxidase, we performed saturation mutagenesis at these positions and screened for mutants with increased activity against guaiacol. The obtained results yield important insights into how these residues affect the chemistry of CCP and peroxidases in general.

Results

Generation and screening of libraries

The role of Arg48 in the control of reactivity and specificity in CCP was addressed by randomization of the corresponding codon in the gene by saturation mutagenesis and screening of the resulting library CM1 for increased activity against guaiacol by using a colony screen and substrate concentrations of 170 μM H_2O_2 and 5 mM guaiacol.[17] In these and the following experiments, CCP possesses an N-terminal 6 × His tag and is termed hCCP. Each of the 20 amino acids should have been present at position 48 of CCP several times in the screening experiments. 60% of the colonies stained faster than, or at least as fast as, the wild-type colony and five of the fastest-staining colonies were isolated and the CCP genes sequenced. Two clones carried the mutation Arg48Gln, whereas the mutations Arg48His, Arg48Thr, and Arg48Ile were each found once. The relatively high percentage of clones that stained faster than the wild-type species can be considered as an indication that the increase in guaiacol activity of the mutant Arg48His is not a result of general-acid/base catalysis by the histidine residue. Four clones that did not stain at all in the screening assay were analyzed for expression of the peroxidase by SDS gel electrophoresis. One of these four clones did not express the full-length protein, the three others were analyzed by DNA sequencing. In all three clones the mutation Arg48Ser was found, although they all possessed different codons (TCT, TCC, AGT).

Simultaneous saturation mutagenesis of Arg48, Trp51, and Trp191 was performed to give library CM2, which was used to investigate the influence of Trp51 and Trp191 on the reactivity of CCP. Arg48 was included in these experiments to identify possible synergistic effects between the residues. In order to identify mutants that possess both high activity against guaiacol and high stability of the reactive enzyme intermediates, we increased the hydrogen peroxide concentration nearly 60-fold and also added ascorbate to the screening solution. Ascorbate scavenges the initially generated phenoxyl radicals and thus the brown tetraguaiacol that results from secondary reactions of these radicals and is responsible for the staining of the colonies is only formed when all the ascorbate has been consumed.[24] Approximately 7% of the colonies stained under these conditions. After screening a total of 15000 mutants, 12 of the fastest-staining mutants were analyzed by sequencing (Table 1). The distal arginine residue at position 48 is conserved in 11 out of 12 mutants. The conservation of the distal arginine residue in these experiments as opposed to its mutation in the screening of library CM1 is caused by the changed screening conditions. For example, the mutant RFS led to faster staining of colonies than the mutant Arg48Gln under screening conditions that favor both activity and stability (that is, the conditions used for the screening of library CM2), but the staining order reverses under

Table 1. *Clones isolated from library CM2.*

Clone[a]	Position			Clones/codons[b]
	48	51	191	
CCP	Arg	Trp	Trp	
RFS	Arg	Phe	Ser	6/3
RFR	Arg	Phe	Arg	3/2
RFC	Arg	Phe	Cys	2/1
KWH	Lys	Trp	His	1/1

[a] Mutants are abbreviated to the one-letter codes of the amino acids at positions 48, 51, and 191. [b] Number of clones isolated/number of clones with different codons.

screening conditions that favor only high reactivity (that is, the conditions used for the screening of library CM1).

The tryptophan residue at position 51 of CCP is mutated to phenylalanine in 11 out of 12 clones and conserved in one case. Within the superfamily of peroxidases, only phenylalanine or tryptophan residues are found at this position.[1] Surprisingly, Trp191 is not conserved in any of the isolated mutants and either small hydrophilic or positively charged amino acids are found at this position.

Expression and characterization of the selected mutants

The selected mutants from libraries CM1 and CM2 were expressed and purified. For comparison, the mutants Trp51Phe and KFH were also prepared. The UV spectra of the mutants from library CM1 displayed some variation. While Arg48Gln and Arg48His displayed UV spectra basically identical to hCCP, Arg48Thr and Arg48Ile showed a broader Soret band and Arg48Thr displayed an additional shoulder at 427 nm (data not shown).[6, 25] Furthermore, the heme-content of these mutants was only 60% (Arg48Ile) and 80% (Arg48Thr) of the value for hCCP, although an excess of heme was present during the reconstitution of the peroxidase. No further attempts were undertaken to increase the heme content of these two mutants. In all following experiments, the calculated concentration of the mutants was based on the heme content of the purified peroxidase. The UV spectra of the mutants isolated from library CM2 at pH 6 were also measured (data not shown). Those mutants with an arginine residue at position 48 displayed a Soret band at 408 – 409 nm as well as the typical α, β and charge-transfer bands that are characteristic for a five-coordinated high-spin ferric heme group.[6, 25] In contrast, the mutant KWH, which possesses a lysine residue at position 48, has a Soret band shifted to 413 nm and a blue-shifted charge-transfer band. Similar spectroscopic properties have been reported for the single mutant Arg48Lys and have been assigned to a six-coordinated high-spin ferric heme group.[18]

In order to investigate whether one of the mutants from library CM2 possesses a stable porphyrin π cation radical as observed for HRP, the mutants were incubated with 1.5 equivalents H_2O_2 and a UV spectrum was recorded immediately after mixing (Figure 2). A spectrum comparable to that of compound I of hCCP with the Soret band shifted to around 420 nm and two new charge-transfer bands at around 530 nm and around

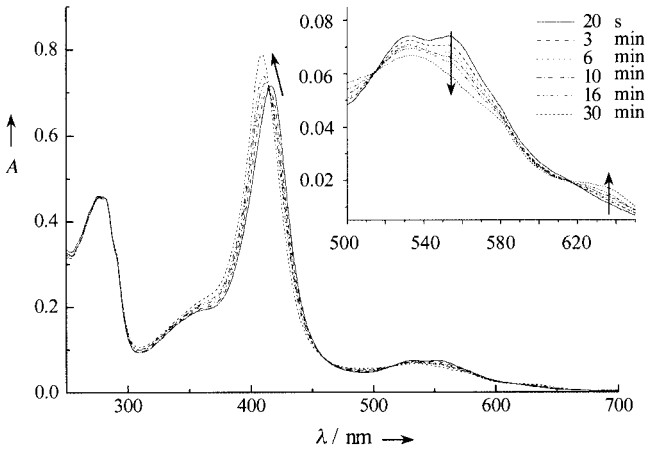

Figure 2. *Formation and decay of compound I of the CCP mutant RFS after mixing of the enzyme with H_2O_2. The blue shift and increase in intensity of the Soret band, the decrease in absorbance of the characteristic band around 560 nm, and the appearance of a charge-transfer band at 637 nm are indicated.*

558 nm was obtained for all mutants, which indicates that a protein radical is formed, as with wild-type hCCP.[6, 26] It has been observed for the Trp191Phe mutant that the porphyrin π cation radical is only transient and decays within milliseconds to a protein-based cation radical, most likely a tyrosyl radical located at Tyr36, Tyr39, or Tyr42.[16, 27, 28] Compound I is then slowly converted to an unidentified species for which the Soret band is shifted back to around 410 nm (Figure 2).

Peroxidase activity of the mutants isolated from libraries CM1 and CM2

The activities of the mutants against the two phenolic substrates guaiacol and pyrogallol as well as horse heart ferrocytochrome *c* and the common peroxidase substrates 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferrocyanate were measured (Table 2).

The activities against guaiacol of all of the selected mutants isolated from library CM1 were significantly increased compared to that of the wild-type protein, with the Arg48Gln mutant about 300-fold more active than the wild-type enzyme. Furthermore, the activities against another phenolic substrate, pyrogallol, were also increased, with the Arg48Gln mutant 50-fold more active than the wild-type enzyme. In contrast, the activities of the selected mutants against the natural substrate ferrocytochrome *c* as well as against ABTS and ferrocyanate were much less affected and are, in general, similar to or slightly below the activity of the wild-type enzyme. The selected Arg48 mutations thus specifically increase the activity against phenols and the mutant Arg48Gln is even more active than the mutant Arg48His. These results strongly argue against the possibility that a histidine residue at position 48 might act as a general-acid/base catalyst but support the hypothesis that these mutations increase the steric access to the ferryl oxygen atom.[17]

In addition, all four Arg48 mutants showed, to varying degrees, inhibition of guaiacol oxidation at higher substrate concentrations and none of them display typical Michaelis –

Table 2. *Kinetic parameters of the selected mutants for the oxidation of various substrates.*[a]

Clone	Guaiacol k_{obs} [sec^{-1}]	Pyrogallol k_{obs} [sec^{-1}]	Cytochrome *c* k_{obs} [sec^{-1}]	ABTS k_{obs} [sec^{-1}]	$K_4Fe(CN)_6$ k_{obs} [sec^{-1}]	Thioanisole k_{obs} [min^{-1}]
hCCP	0.35 ± 0.03	2.3 ± 0.3	676 ± 4	3.3 ± 0.1	212 ± 3	0.5 ± 0.2
Arg48Gln	102 ± 5	104 ± 5	689 ± 43	2.3 ± 0.1	270 ± 7	2.9 ± 0.1
Arg48His	26 ± 2	89 ± 2	388 ± 99	0.8 ± 0.1	124 ± 4	2.5 ± 0.4
Arg48Ile	13 ± 7	7.6 ± 1.5	138 ± 9	0.8 ± 0.1	66 ± 4	4.1 ± 1.1
Arg48Thr	5 ± 0.3	57 ± 4	216 ± 7	4.5 ± 0.5	136 ± 3	3.5 ± 0.2
RFS	13.8 ± 0.1	50.1 ± 3.3	< 0.1%	0.8 ± 0.1	n.d.	n.d.
RFR	8.92 ± 0.1	13.8 ± 0.5	< 0.1%	0.5 ± 0.1	n.d.	n.d.
RFC	6.11 ± 0.3	16.7 ± 2.4	< 0.1%	1.0 ± 0.1	n.d.	n.d.
KWH	21.2 ± 4.5	10.2 ± 0.4	< 0.1%	0.9 ± 0.1	n.d.	n.d.
Trp51Phe	2.8 ± 0.04	13.0 ± 1.4	1460 ± 190	3.0 ± 0.1	81 ± 5	n.d.
Trp191Ser	0.44 ± 0.02	4.0 ± 0.3	< 0.1%	0.8 ± 0.1	n.d.	n.d.
KFH	0.23 ± 0.04	0.3 ± 0.1	< 0.1%	0.6 ± 0.1	n.d.	n.d.

[a] Double and triple mutants are abbreviated to the one-letter codes of the amino acids at positions 48, 51, and 191. n.d. = not determined

Menten kinetics (Figure 3). This inhibition is absent in wild-type hCCP and in the mutant Trp51Phe (Figure 3), but has also been observed for the previously selected mutants that possess, in addition to various other mutations, the mutation Arg48His.[17] A possible explanation is that the inhibition is caused by improved

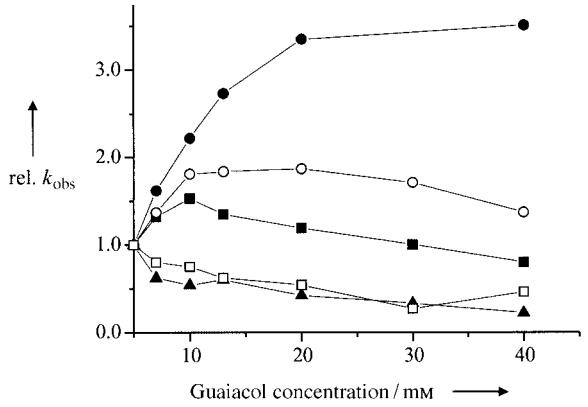

Figure 3. *Relative observed rate constants (rel. k_{obs}) of Arg48Gln (■), Arg48His (○), Arg48Ile (□), Arg48Thr (▲), and Trp51Phe (●) mutants for the oxidation of guaiacol as a function of the guaiacol concentration. The rel. k_{obs} values were obtained by division of k_{obs} at a given concentration by the rate constant obtained with 5 mM guaiacol.*

binding of the substrate in the less polar distal cavity of the mutants and resulting inhibition of compound I formation at high guaiacol concentrations.[17, 29] The fact that all selected Arg48 mutants with increased activity against guaiacol show inhibition of this activity at high substrate concentrations supports this hypothesis.

All mutants selected from library CM2 possessed an increased activity against guaiacol and pyrogallol. In contrast, none of the mutants possessed an increased activity against ABTS. This indicates that the selected mutations also affect foremost the activity against phenols. As can be expected for mutation of Trp191, which plays an important role in the electron transfer from cytochrome *c* to CCP, none of the selected mutants possess a significant activity against cytochrome *c*.[30] The mutants Trp51Phe and Trp191Ser were prepared and their activities determined (Table 2) in order to investigate the relative contributions of the mutations at positions 51 and 191 to the increased peroxidase activity. As described in the literature, the mutation Trp51Phe significantly increased the activity against both phenols and, to a less extent, against cytochrome *c*.[14–16] The effect of the mutation Trp191Ser on the activity against phenols was less significant but abolished the activity against cytochrome *c* (Table 2). This latter result is in accordance with data published on the mutant Trp191Phe.[30] The effect of mutations at positions 48, 51, and 191 are not necessarily additive. For example, introduction of the mutation Trp191Ser into the mutant Trp51Phe to yield double mutant RFS increases the activity against phenols fivefold, whereas the Trp191Ser mutation alone has no significant effect on wild-type CCP. A further example of synergistic effects in CCP can be found in mutant KWH. The double mutation Arg48Lys and Trp191His in KWH increases the activity of CCP against guaiacol 60-fold. Introduction of the additional mutation Trp51Phe into this double mutant (to yield mutant KFH), a mutation that by itself increases the activity against guaiacol eightfold, diminishes the activity against guaiacol to a level below that of hCCP (Table 2).

A comparison of the guaiacol activity of the mutants isolated from the different libraries shows that the Arg48Gln mutant is at least seven times more active than any mutant isolated from library CM2. In order to explain the observation that Arg48 was conserved in the screenings performed with library CM2, we argued that the stability of the reactive intermediates plays a more pronounced role in those experiments than in the CM1 experiments. To verify this hypothesis and to investigate the relative stability of the different mutants, the mutants isolated from library CM2, as well as Arg48Gln and hCCP, were preincubated with 10 mM H_2O_2 in the absence of substrate and the activity of the peroxidase was measured as a function of time of preincubation with H_2O_2 (Figure 4). Mutant Arg48Gln lost most of its activity within one minute, whereas the mutants isolated from library CM2 are more stable under these conditions and already possess a higher residual activity than Arg48Gln after one minute of incubation. In particular, the mutant RFS maintained its full activity even after 14 minutes of incubation in 10 mM H_2O_2 and thus possesses a stability comparable to hCCP under these conditions.

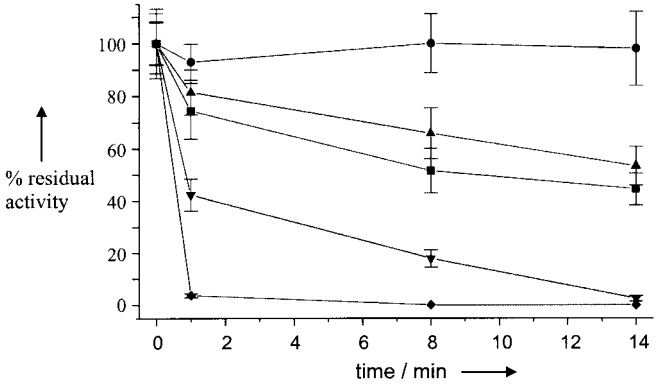

Figure 4. Residual activity of selected mutants against guaiacol after preincubation in H_2O_2 (10 mM). RFR (■), RFS (●), RFC (▲), KWH (▼), Arg48Gln (◆). hCCP does not show any inactivation under these conditions (data not shown).

Formation and stability of compound I from Arg48Gln and Arg48His mutants

The mutations at Arg48 had the highest influence on the stability of compound I. The rate of formation and the stability of compound I of the Arg48His and Arg48Gln mutants were investigated in detail by analysis of the reaction of these mutants with H_2O_2 by using stopped-flow techniques (Figure 5). Formation of compound I shifts the maximum of the Soret band to higher wavelengths and its formation and decay in the absence of substrate can be followed by measurement of the absorbance change at 424 nm.[26, 27]

A UV spectrum characteristic for compound I was observed for both mutant enzymes 1.5 sec after mixing of the enzyme with H_2O_2: the Soret band of both mutants is red shifted to approximately 420 nm and two new charge-transfer bands at approximately 533 and 557 nm appear (Figure 5).[26, 27] The formation of compound I could be best described by a single-exponential function that depends linearly on the H_2O_2 concentration (Figure 5). The resulting second-order rate constants for the Arg48Gln and Arg48His mutants, $(11.7 \pm 0.3) \times 10^6\ s^{-1}\,M^{-1}$ and $(9.5 \pm 0.3) \times 10^6\ s^{-1}\,M^{-1}$, respectively, were only a factor of 3 – 4 below that of the wild-type enzyme.[31] The relatively small effect of the mutation Arg48Gln on the rate of compound-I formation indicates that the positive charge of the guanidinium group of the distal arginine residue does not significantly contribute to the stabilization of the negative charge that develops in the course of the heterolytic cleavage of the peroxide bond. The following decrease in absorbance in the stopped-flow experiments can be attributed to the decay of compound I (Figure 5). For both mutants, this decay can be best fitted to a two-exponential function. Between 5 and 15 μM H_2O_2, the rate constants showed no significant dependence on the H_2O_2 concentration and are given as mean values of the first-order rate constants obtained in this concentration range. The corresponding rate constants for the Arg48Gln mutant are $(3.8 \pm 0.4) \times 10^{-3}\ s^{-1}$ (amplitude of 60%) and $(1.1 \pm 0.4) \times 10^{-3}\ s^{-1}$ (amplitude of 40%). Rate constants of $(0.25 \pm 0.08)\ s^{-1}$ (amplitude of 20%) and $(1.0 \pm 0.2) \times 10^{-2}\ s^{-1}$ (amplitude of 80%) were obtained for the Arg48His mutant. The decay of compound I of wild-type CCP has been fitted to a single-exponential function with a rate constant of $(3.0 \pm 0.3) \times 10^{-5}\ s^{-1}$.[26] A comparison of the rate constants of the fast step of the decay leads to the conclusion that compound I of the Arg48Gln mutant is about 65 times more stable than compound I of mutant Arg48His, but still a factor of 10^2 less stable than that of the wild-type enzyme.

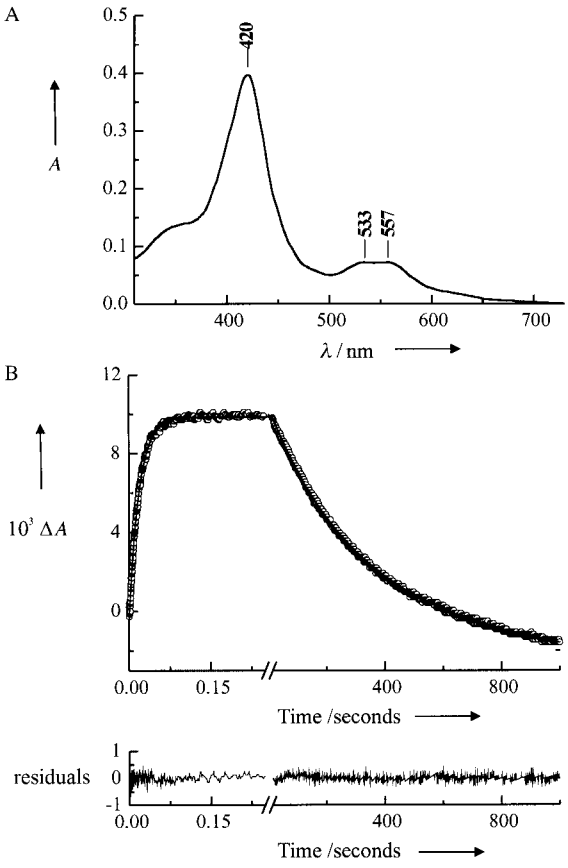

Figure 5. Stopped-flow experiments with Arg48Gln. A) Compound I spectrum of mutant Arg48Gln obtained 1.5 sec after mixing of the peroxidase and H_2O_2. B) Stopped-flow trace at 424 nm observed upon mixing mutant Arg48Gln (1 μM) and H_2O_2 (10 μM) in KH_2PO_4 (50 mM, pH 6) at 25 °C. The transient spectra were measured four times and averaged. The solid curve represents the best fit to a three-exponential function.

Peroxygenase activity of the mutants Arg48Gln, Arg48His, Arg48Ile, and Arg48Thr

The specific increase in activity of the selected mutants against phenols suggests that Arg48 controls access to the ferryl oxygen atom and the distal cavity in general. The peroxygenase activity of these mutants was investigated to independently test this hypothesis. The peroxygenase activity of peroxidases is mainly influenced by mutations that increase access to the ferryl oxygen atom and thus can be considered as a measure of the accessibility of the ferryl species in the distal cavity of peroxidases.[21, 32–35] Thioanisole is oxidized by peroxidases to the corresponding *S*-oxide and it has been shown that the ferryl oxygen atom of compound I of CCP and HRP is directly

transferred to the thioether.[32, 34, 35] The reaction of the mutants with thioanisole was followed at pH 7 with 1 mM thioanisole and 1 mM H_2O_2. Thioanisole and the corresponding *S*-oxide were quantified by HPLC. Under the applied conditions, the background peroxygenation was about 50% of the reaction catalyzed by the wild-type enzyme and was subtracted from all measured rates. Rapid inactivation of all mutants was observed. The wild-type enzyme still possessed about 65% of its initial activity after 3 min but all of the mutants were completely inactivated within 3 min and mutants Arg48His, Arg48Thr, and Arg48Ile even within one minute. The reaction was therefore quenched after 1 min and analyzed by HPLC. As can be seen in Table 2, all of the mutants catalyzed the sulfoxidation of thioanisole more efficiently than the wild-type enzyme. The conclusion that the selected mutations at position Arg48 increase the access to the ferryl oxygen atom is thus supported by the increased activity of all isolated mutants in the peroxygenation of thioanisole, even though the activities of the mutants are hampered by their rapid inactivation under these conditions.

Discussion

The implications of the performed combinatorial mutagenesis experiments for our understanding of the role of the residues Arg48, Trp51, and Trp191 in the control of the reactivity and selectivity of CCP will be discussed for each residue individually.

The conservation of the distal arginine residue under conditions that demand both stability and reactivity and its mutation under conditions that favor high reactivity points towards a dual role of this residue in control of reactivity and in stabilization of the reactive intermediate compound I. Careful choice of conditions in the directed evolution experiments allows selection of one of these traits at the expense of the other. The influence of the distal arginine residue on the stability of compound I can be best seen in the stopped-flow experiments with mutants Arg48Gln and Arg48His. Despite being the most stable Arg48 mutant isolated so far, Arg48Gln is 2 orders of magnitude less stable than compound I of wild-type CCP. The relative stability of the Arg48Gln mutant could be rationalized by the argument that the amide nitrogen atom of a glutamine side chain could, to some extent, act as a substitute for the N^{ε} atom of Arg48 and donate a hydrogen bond to the ferryl oxygen atom, thereby stabilizing compound I. Furthermore, the observed flexibility of the Arg48 side chain in the distal cavity should also allow the glutamine residue to adopt a conformation that enables the formation of a hydrogen bond to the ferryl oxygen atom.[35–37] The relatively small effect of the mutation Arg48Gln on the rate of compound I formation, which shows a second-order rate constant comparable to wild-type CCP and the previously described Arg48Lys mutant, indicates that the positive charge of the guanidinium group does not significantly contribute to the stabilization of the negative charge that develops in the course of the heterolytic cleavage of the peroxide bond.[18]

The influence of mutations of the distal arginine residue of CCP on the reactivity of the peroxidase is highlighted by the 300-fold increase in activity of the Arg48Gln mutant towards guaiacol compared to the wild-type enzyme. The higher activity of the Arg48Gln mutant compared to the Arg48His mutant shows that a histidine residue at this position does not act as a general-acid/base catalyst in phenol oxidation and supports the earlier hypothesis that mutations of the distal arginine residue increase the steric access to the ferryl oxygen atom.[17] Furthermore, the increased steric access to the ferryl oxygen atom in the selected Arg48 mutants is independently demonstrated by the increased peroxygenase activity of all these mutants, an activity which can be considered as a measure of the accessibility of the ferryl oxygen atom of compound I.[21, 32–35] In agreement with these data, the mutations at Arg48 do not significantly affect those activities that do not require access to the ferryl group. The N^{ε} atom of Arg48 is reoriented towards the ferryl oxygen atom in the crystal structure of compound I of CCP in comparison to the crystal structure of the ferric enzyme, which indicates the presence of a strong hydrogen bond.[12] Removal of this strong hydrogen bond or replacment by a weaker bond might thus facilitate proton transfer from the phenol group to the ferryl oxygen atom and contribute to the observed increase in activity against these substrates. The relatively small effect of the removal of the hydrogen bond between Arg48 and the ferryl oxygen atom on the activity against other peroxidase substrates such as ABTS and ferrocyanide indicates that the hydrogen bond itself is not very important for the general reactivity of the peroxidase. Clearly, structural information on the mutants would allow a more detailed discussion of our data. Such information would also allow further questions to be addressed, such as why the mutation Arg48Gln gives the most active of the selected Arg48 mutants, or why the Arg48Ser mutant fails to form a functional peroxidase while the mutation Arg48Thr increases peroxidase activity.

In summary, our data on the distal arginine residue of CCP show that this residue is not only important for stabilization of compound I but also controls the reactivity and specificity of the peroxidase by restriction of access to the ferryl oxygen atom. This dual role of the distal arginine residue probably also contributed to the conservation of this residue in the superfamily of bacterial, plant, and fungal peroxidases.

Our data for Trp51 show that either a phenylalanine or a tryptophan residue is required at position 51 of CCP and that the removal of the hydrogen bond between the indol ring of Trp51 in CCP and the ferryl oxygen atom mostly affects those reactions that involve proton transfer from the substrate to the ferryl oxygen atom, such as the oxidation of phenols. The significant increase in activity against phenols observed can be rationalized by considering that the removal of the hydrogen bond between Trp51 and the ferryl oxygen atom will increase the affinity of the ferryl oxygen atom towards other hydrogen donors, either active-site water molecules or phenol groups, and should thus make the pathway for proton transfer from the phenol to the ferryl oxygen atom more efficient. However, at least one of the two hydrogen donors that form hydrogen bonds with the ferryl oxygen atom of compound I and compound II, either Arg48 or Trp51, is conserved in all selected mutants and the removal of both hydrogen donors leads to a drastic drop in activity against

guaiacol. Furthermore, the perfect stacking of the aromatic ring of either a tryptophan or a phenylalanine residue with the heme group is expected to significantly contribute to the affinity of the peroxidase for the cofactor (Figure 1). This stacking effect should contribute to the conservation of an aromatic residue at this position as the apoenzyme must compete with other heme-containing enzymes for binding of the cofactor in vivo and mutations that decrease or increase the affinity of the peroxidase for the cofactor affect the in vivo concentration of active holoenzyme.[17]

In contrast to Trp51, Trp191 of CCP can be replaced by a variety of polar or positively charged residues. This observed variability inside the core of the protein, in particular the introduction of a positive charge, can be interpreted as a reflection of the mechanism of the peroxidase.[8] In the mechanism of CCP, Trp191 is oxidized to a transient indolyl cation radical and it can be expected that those interactions that stabilize the cation radical will also stabilize a positively charged or polar residue within the core of the protein. The stabilization of compound I of CCP by a cation binding motif has been shown.[38] In addition, the binding of small positively charged organic ligands in the cavity created by the mutation Trp191Gly shows that Trp191 is surrounded by a rather polar environment.[39, 40] Furthermore, the coexistence of so-called closed and open conformations of CCP, for which Trp191 is surface exposed in the open form, has been reported.[41] Although only 4% of the wild-type enzyme is in the open conformation at any given time, the existence of this conformation further underlines the degree of structural flexibility within this functionally important region. The formation of relatively stable protein-based radicals by the Trp191 mutants selected here supports the idea that the alternative radical site(s) used might also contribute to the relative stability of compound I of the wild-type enzyme.[16] Those factors that are responsible for the formation of a stable porphyrin π cation radical still have to be identified and it remains to be determined how these factors affect the activity and selectivity of the peroxidases.

Experimental Section

Reagents: Chemicals were purchased from Fluka AG. Enzymes for recombinant DNA work were purchased from MBI Fermentas or New England Biolabs. Ni-NTA-agarose (Qiagen) was used for the affinity purification. Libraries were plated on Hybond-C super membranes (Amersham Life Sciences).

Construction and screening of libraries and expression of selected clones: The previously described plasmid phCCP was used for the construction and screening of CCP libraries as well as the expression of individual mutants.[17] In this plasmid, CCP possesses an N-terminal $6\times$ His tag and is termed hCCP. hCCP mutants were expressed and purified as previously described.[17] Heme content was determined by the pyridine hemochromogen assay.[42] Protein concentrations were measured by determination of the absorbance at 280 nm by using an extinction coefficient of $\varepsilon = 74\ \mathrm{mM^{-1}\,cm^{-1}}$.[43] Purity of the recombinant enzymes was checked by SDS gel electrophoresis and was greater than 95%. Splice overlap extension (SOE) PCR was performed, with appropriate primers, for the saturation mutagenesis at position Arg48 (library CM1) and the simultaneous saturation mutagenesis at Arg48, Trp51, and Trp191 (library CM2).[44] Pfu DNA polymerase was used in these experiments. The PCR product was digested with *Sfi*I and ligated into the *Sfi*I-digested plasmid phCCP. The sizes of the two libraries CM1 and CM2 after electroporation into BL21(DE3) cells were 1.5×10^4 and 2×10^4 independent clones, respectively. Individual mutants were also prepared by using SOE PCR.[44]

Both libraries were directly grown on a Hybond-C super membrane (Amersham Life Sciences) placed on an LB agar plate (100 mg L^{-1} ampicillin, 1 mM isopropyl-β-D-1-thiogalactopyranoside).[17] Up to 4000 colonies were grown per membrane. The membranes with the colonies were placed on filter paper soaked with substrate solution for the screening. Screening of library CM1 was performed at 25 °C with guaiacol (5 mM) and H_2O_2 (170 μM) in KH_2PO_4 buffer (50 mM, pH 6). Screening of library CM2 was performed at 25 °C with guaiacol (20 mM), ascorbate (0.2 mM), and H_2O_2 (10 mM) in KH_2PO_4 buffer (50 mM, pH 6). In total, about 2000 colonies of CM1 and 15 000 colonies of CM2 were screened. Active clones were identified by visual inspection of the membrane for the staining of colonies by the brown color of the product of guaiacol oxidation.[45] The fastest-staining colonies were re-amplified in LB medium (100 mg L^{-1} ampicillin).

Kinetic measurements: The kinetics of the guaiacol, pyrogallol, $K_4Fe(CN)_6$, and cytochrome *c* oxidation reactions were measured in KH_2PO_4 (50 mM, pH 6) at 30 °C as described previously.[17] The concentrations used were: guaiacol, 5 mM; pyrogallol, 5 mM; cytochrome *c* from horse heart, 40 μM; $K_4Fe(CN)_6$, 17 mM. ABTS measurements were taken at 30 °C on a microtiter plate reader (Molecular Devices Spectra Max 340) in KH_2PO_4 (50 mM, pH 6), bovine serum albumin (0.1 mg ml^{-1}), H_2O_2 (250 μM), and ABTS (0.25 mM) and the absorbance at 414 nm ($\varepsilon = 36\ \mathrm{mM^{-1}\,cm^{-1}}$) was recorded.[46] The observed first-order rate constants were obtained by division of the measured reaction velocity by the enzyme concentration.

Stopped-flow kinetics were measured with an Applied Photophysics SX 18-MV stopped-flow spectrometer in KH_2PO_4 (50 mM, pH 6) at 25 °C. The formation and decay of compound I were monitored at 424 nm and at H_2O_2 concentrations between 5 and 15 μM. The concentration of the enzyme was 0.25 μM after mixing. Spectra of compound I were recorded on a J&M TIDAS diode array spectrometer.

The peroxygenation of thioanisole was measured at pH 7 in KH_2PO_4 (50 mM) and methanol (5% v/v) with thioanisole (1 mM), peroxidase (18 μM), and H_2O_2 (1 mM) at 25 °C. The reaction was quenched after 1 min by addition of hydroxylamine to a concentration of 50 mM. 3-Nitrobenzyl alcohol was added to a concentration of 50 mM as a standard before extraction of the mixture twice with equal volumes of CH_2Cl_2. The solvent was evaporated and the residue dissolved in a mixture of acetonitrile and water (100 μL, 2:3). The samples were centrifuged for 5 min and analyzed by HPLC on a Nucleosil 100–5 C18 AB column (Macherey & Nagel). A gradient from 10% acetonitrile in water to 70% acetonitrile over 10 min was used. Peaks were detected with a dual wavelength absorbance detector (Waters HPLC system 2790) at 252 nm and 230 nm. The k_{obs} values of the mutants in Table 2 also correspond to the turnover of thioanisole molecules per enzyme in the assay.

Funding of this work was provided by the Swiss Science Foundation and the European Community (Grant no. BMH4-CT97–2277). We thank Prof. André E. Merbach for support in the stopped-flow experiments.

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Received: May 7, 2002 [F 410]