Directed Molecular Evolution of Cytochrome *c* Peroxidase†

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ABSTRACT: Cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* was subjected to directed molecular evolution to generate mutants with increased activity against the classical peroxidase substrate guaiacol, thus changing the substrate specificity of CCP from the protein cytochrome *c* to a small organic molecule. After three rounds of DNA shuffling and screening, mutants were isolated which possessed a 300-fold increased activity against guaiacol and an up to 1000-fold increased specificity for this substrate relative to that for the natural substrate. In all of the selected mutants, the distal arginine (Arg48), which is fully conserved in the superfamily of peroxidases, was mutated to histidine, showing that this mutation plays a key role in the significant increase in activity against phenolic substrates. The results suggest that, in addition to stabilizing the reactive intermediate compound I, the distal arginine plays an important role as a gatekeeper in the active site of CCP, controlling the access to the ferryl oxygen and the distal histidine. Other isolated mutations increase the general reactivity of the peroxidase or increase the intracellular concentration of the active holo form, allowing their selection under the employed screening conditions. The results illustrate the ability of directed molecular evolution technologies to deliver solutions to biochemical problems that would not be readily predicted by rational design.

An intriguing problem in biochemistry is the question of how the protein scaffolds in heme-containing enzymes control the reactivity of the cofactor. To identify residues that play an important role in controlling the reactivity of one of the best-characterized peroxidases, the cytochrome *c* peroxidase (CCP)[1] from *Saccharomyces cerevisiae*, we have decided to use directed molecular evolution to generate mutants with novel substrate specificities. CCP is a mitochondrial, heme-containing peroxidase that catalyzes the oxidation of ferrocytochrome *c* by H_2O_2 (*1*). It is a monomeric protein of 294 residues and is a member of the superfamily of plant, fungal, and bacterial peroxidases (*2*). In addition to CCP, members of this superfamily include the bacterial catalase peroxidases, ascorbate peroxidases (AP), and secretory fungal and plant peroxidases such as *Coprinus cinereus* peroxidase (CIP) and horseradish peroxidase (HRP). The first step in the reaction mechanism of these peroxidases is their reaction with H_2O_2 which forms an oxyferryl (Fe^{4+}═O) species and an organic cation radical, the so-called compound I (*1*). In CCP, the organic cation radical is an indolyl cation radical at Trp191 (*3*), whereas the other peroxidases of this superfamily form a porphyrin π cation radical. Compound I of CCP then oxidizes one molecule of ferrocytochrome *c* (Cc^{2+}) to ericytochrome *c* (Cc^{3+}), it thereby being converted to an oxyferryl (Fe^{4+}═O) species, which is called compound II (*1*). Compound II subsequently reacts with another molecule of ferrocytochrome *c*, converting CCP back into its resting state (*1*).

The substrate specificities within this superfamily of peroxidases vary considerably, with substrates ranging from a protein in the case of CCP to classical peroxidase substrates such as phenols and anilines in the case of HRP and CIP (*4*). Furthermore, bacterial catalase-peroxidases possess significant catalase activity (*5*). In addition to its natural substrate, CCP can oxidize small organic and inorganic substrates, although its activity is orders of magnitude below that of HRP or CIP and the small substrates are oxidized at different sites of CCP than cytochrome *c* (*6*). Small substrates approach the heme from its distal side, and the electron transfer is believed to take place near the δ-meso edge of the porphyrin (*6*). Within the formed complex of cytochrome *c* and CCP, the two hemes are more than 17 Å apart and the electron transfer is believed to occur via the proximal side of the heme of CCP (*7*).

CCP has been shown to be an ideal system for probing and manipulating the properties of a heme enzyme. For example, there have been various efforts to rationally change the enzymatic properties of CCP, including the design of mutants with increased or altered specificity against small peroxidase substrates (*8*, *9*), the introduction of metal binding sites (*10*, *11*), the transformation of CCP into a manganese peroxidase (*12*, *13*), or the generation of mutants capable of oxidizing hydroxyarginine (*14*). Despite these successes, these studies also show that, due to the complexity of the protein−cofactor interactions, a detailed understanding of all the mechanistic questions as well as the rational design of

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of oxidation state; CCP, cytochrome *c* peroxidase; hCCP, CCP with an N-terminal six-His tag; HRP, horseradish peroxidase isozyme *c*; CIP, *C. cinereus* peroxidase; AP, ascorbate peroxidase; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); IPTG, isopropyl β-D-1-thiogalactopyranoside; Amp, ampicillin.

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peroxidases or heme enzymes with tailor-made activities is still a very ambitious goal. As an alternative to rational design, the directed molecular evolution of enzymes is a very powerful tool for generating mutants with novel properties for a particular application or for studying enzyme mechanisms (*15*). Recently, the directed molecular evolution of heme proteins has been used to study their structure−function relationship as well as to develop mutants for practical applications. These experiments include the generation of CIP mutants with increased thermal and oxidative stability (*16*), the generation of HRP mutants with better folding properties (*17*), the isolation of myoglobin mutants with increased peroxidase activity (*18*), the increase of the thermostability of a catalase-peroxidase (*19*), and the selection of cytochrome P450 enzymes with improved properties as biocatalysts (*20*).

We describe here our experiments aimed at establishing a system for the directed molecular evolution of CCP and its application in generating mutants with novel substrate specificities. Using this system, CCP mutants with a 300-fold increased activity against the classical peroxidase substrate guaiacol were generated. Surprisingly, in all of the highly active mutants Arg48, which is one of only nine residues that are fully conserved in the superfamily of plant peroxidases (*2*), has been mutated to histidine.

EXPERIMENTAL PROCEDURES

Reagents. Chemicals used in this work were purchased from Fluka AG. Enzymes for recombinant DNA work were purchased from MBI Fermentas or New England Biolabs. DNase I (from bovine pancreas, cell culture grade) was purchased from Boehringer Mannheim. Cytochrome *c* (from horse heart) was purchased from Fluka. For the affinity purification, Ni−NTA−agarose (Qiagen) was used. Libraries were plated on *Hybond-C super* membranes (Amersham Life Sciences).

Oligonucleotides. The following primers were used: AI3461, 5′CGATATAGGCGCCAGCAACC3′; AI3462, 5′AAGCTTTAATGCGGTAGTTTATCACAGT3′; AI3771, 5′CGATGCGTCCGGCGTAGAG3′; AI3772, 5′CACCGTCACCCTGGATGCTGTA3′; PT01, 5′ATATCGATATGGCCAGCACGGCCACACCGCTCGTTCATGT3′; and HS057, 5′CCGTAGGTACCGGCCAAAAAGGCCTCACTATAAACCTTGTTCC3′.

Construction of a Vector for Expression and Screening of Libraries. The CCP gene, from Thr1 to Leu294, was amplified by PCR from *S. cerevisiae* using primers PT01 and HS057, digested with *Sfi*I, and ligated into a *Sfi*I-digested pET-15b derivative (*21*). In the resulting plasmid phCCP, the CCP gene is downstream of the T7 promoter, possesses an N-terminal six-His tag, and is placed between two different *Sfi*I sites, which allow for the efficient directional cloning of CCP libraries into the vector. One of the two *Sfi*I sites lies between the six-His tag and Thr1, and the other immediately after the stop codon. In this construct, the following amino acids are expressed as an N-terminal fusion to CCP: MSSHHHHHHSSAMASTA. The sequence of the construct was verified by sequencing.

Expression of Wild-Type hCCP and Mutants. Escherichia coli Bl21(DE3) cells carrying plasmid phCCP were grown in 50 mL of LB (100 μg/mL Amp) at 37 °C and 190 rpm until the OD_{600} reached 0.6 and, after addition of IPTG (1 mM final concentration), incubated for an additional 3 h at 37 °C. Bacteria were collected by centrifugation, resuspended in 3 mL of lysis buffer [50 mM NaH_2PO_4, 5 mM imidazole, and 300 mM NaCl (pH 8)] with 3 mg/mL lysozyme and kept on ice for 30 min. Extended sonification at this point, probably due to the solubilization of inclusion bodies, increased the yield of soluble hCCP. The lysate was centrifuged at 10000*g* and 4 °C for 10 min and the pH of the supernatant adjusted to pH 7. Hemin was added to a final concentration of 200 μM, and the solution was kept at 4 °C for 5 min. Ni−NTA−agarose (350 μL), equilibrated with lysis buffer, was added and the resulting solution mixed by inversion for 5 min. The capacity of the resin at this point was smaller than the amount of hCCP. The resin was poured into a 5 mL polypropylene column, washed with 3 mL of washing buffer [50 mM NaH_2PO_4, 15 mM imidazole, and 300 mM NaCl (pH 7.5)], and the protein was eluted with 1 mL of elution buffer (200 mM imidazole in washing buffer). The eluted protein was dialyzed against 50 mM KH_2PO_4 and 65% glycerol (pH 6) at 4 °C and stored in aliquots at −80 °C. The purity was checked by 11% SDS−PAGE and estimated to be around 95%. The ratio A_{408}/A_{280} for wild-type hCCP was 1.22, and those of the mutants were between 1.25 and 1.5. Heme content was determined by the pyridine hemochromogen assay (*22*). Protein concentrations were measured by the Bradford assay (*23*).

Construction of the Libraries and DNA Shuffling. DNA shuffling was performed following the protocol of Stemmer et al. (*24*). A 1.4 kb PCR product using primers AI3461 and AI3462 was amplified from phCCP (encoding either the wild type or mutants selected after screenings) and used for the DNase I fragmentation. Fragments of about 40−100 bp were isolated and reassembled by PCR without primers. Primers AI3771 and AI3772 were then used to amplify a 1.3 kb fragment. The PCR product was digested with *Sfi*I, a 900 bp fragment isolated from a 1% agarose gel, and ligated into *Sfi*I-digested phCCP. The size of the libraries, obtained after electroporation of the ligation product into Bl21(DE3) cells, was usually about 5×10^4 independent clones. The libraries were stored in 10% glycerol as aliquots at −80 °C. The DNA shuffling for the preparation of library PTI was performed with DNA fragments of about 100−400 bp, obtained after digestion of the CCP gene in the presence of Mn^{2+} (*25*). *Error prone* PCR was used to generate library PTII, following the protocol of Arnold et al. and using primers AI3771 and AI3772 (*26*).

Screening of the Libraries. About $3-5 \times 10^3$ independent clones were directly spread on a nitrocellulose membrane (14 cm diameter) and grown on an LB agar plate (100 μg/mL Amp) overnight at 28 °C. The membrane was then placed on an LB agar plate (100 μg/mL Amp) containing 1 mM IPTG and the plate incubated for 3 h at 37 °C. After the incubation, the membrane was briefly air-dried and placed on several layers of filter paper, which were wetted with the substrate and H_2O_2 dissolved in the appropriate buffer. The filter was then visually inspected for the development of colored colonies. The fastest staining colonies were manually picked and amplified overnight in LB medium (100 μg/mL Amp). Alternatively, the CCP gene was directly amplified by PCR using primers AI3461 and AI3462.

Kinetic Measurements. All measurements were taken at 30 °C on a Perkin-Elmer Lamda 10 spectrophotometer if

not stated otherwise. Reported data are the average of at least three independent measurements, unless stated otherwise. Oxidation of guaiacol was assessed at pH 6 with 50 mM KH_2PO_4, 170 μM H_2O_2, and 5 mM guaiacol and an enzyme concentration of 500 nM (wild-type) or 5 nM (mutant) by monitoring the absorbance at 470 nm ($\epsilon = 26.6$ mM^{-1} cm^{-1}) (*6*). For the determination of k_{cat} and K_M values, the guaiacol concentration was varied from 5 to 40 mM. Oxidation of pyrogallol was assessed at pH 6 with 50 mM KH_2PO_4, 170 μM H_2O_2, and 5 mM pyrogallol and an enzyme concentration of 500 nM (wild-type) or 5 nM (mutant) by monitoring the absorbance at 470 nm ($\epsilon = 2.64$ mM^{-1} cm^{-1}) (*8*). Oxidation of ferrocytochrome *c* was assessed at pH 6 with 50 mM KH_2PO_4, 180 μM H_2O_2, and 40 μM ferrocytochrome *c* and enzyme concentrations of 500 pM by monitoring the absorbance at 550 nm ($\Delta\epsilon = 18.5$ mM^{-1} cm^{-1}) (*8*). The data reported here are the average of two measurements. Commercial horse heart cytochrome *c* was reduced with dithionite as described by Yonetani et al., and the concentration of the freshly reduced cytochrome *c* was determined from the absorption at 550 nm ($\epsilon = 27.6$ mM^{-1} cm^{-1}) (*44*). Oxidation of $K_4Fe(CN)_6$ was assessed at pH 6 with 100 mM KH_2PO_4, 600 μM H_2O_2, and 17 mM $K_4Fe(CN)_6$ and an enzyme concentration of 75 nM (wild-type) or 37.5 nM (mutant) by monitoring the absorbance at 420 nm ($\epsilon = 1$ mM^{-1} cm^{-1}) (*10*). For the determination of k_{cat} and K_M values, the $K_4Fe(CN)_6$ concentration was varied from 3 to 25 mM.

Stopped-flow kinetics were measured with an Applied Photophysics SX 18-MV stopped-flow spectrometer and were carried out in 50 mM KH_2PO_4 at pH 6 and 25 °C. The formation and decay of compound I were monitored at 424 nm and at H_2O_2 concentrations of 0.25 μM and between 5 and 15 μM. The concentration of the enzyme was 0.25 μM after mixing. To ensure pseudo-first-order conditions at 0.25 μM H_2O_2, a CCP concentration of 2.5 μM was used under these conditions. Spectra of compound I were recorded using a J&M TIDAS diode array spectrometer.

The pH profiles with guaiacol (5 mM) were measured at 30 °C in 50 mM citric acid/citrate buffer (pH 4.5−5.5) and 50 mM potassium phosphate buffer (pH 5.5−8.0). The ionic strength was kept constant at 180 mM using KNO_3. The H_2O_2 concentration was 170 μM.

RESULTS

Expression of Protein and Construction of Libraries. For the molecular evolution of CCP, the gene from *S. cerevisiae* was cloned into a pET-15b derivative that allows for high expression levels in *E. coli*, the one-step purification with the aid of an affinity tag (six-His tag), and the simple cloning of CCP libraries into the vector due to the presence of two flanking *Sfi*I restriction sites. Purification of the resulting polyhistidine-tagged CCP (hCCP) without adding cofactor after lysis results in the isolation of a large excess of the apoenzyme with the presence of about 1% holoenzyme (vide infra). To convert the apo form to the holo form, hemin is directly added to the lysate before the affinity purification. The one-step purification of hCCP yields protein that is about 95% pure. The resulting hCCP possesses an R_Z value of 1.22, which is close to the literature value of 1.25−1.3 for CCP without an affinity tag. The specific activity of hCCP against cytochrome *c* (936 ± 65 s^{-1}) measured under conditions reported in the literature is similar to the value reported for wild-type CCP under these conditions (581 ± 95 s^{-1}) (*10*). The affinity tag thus does not interfere with the activity of the protein and allows the parallel purification of several mutants in 1 day. The yield is about 1 mg of protein per 50 mL of shake flask culture.

Residue No.	2	5	7	25	35	48	87	94	119	148	154	164	180	184	190	194	201	224	226	246	279	282	288
CCP	T	V	V	A	E	R	N	P	M	D	V	N	T	N	P	A	E	D	K	S	D	S	T
AN1			I			H						D	A		L			N					
AN2						H		A				D	A					N					
AN3						H			V				A	Y		T	G	V	R				
AN4						H							A					N			G		
AN5		I				H							A					V				G	
AN6					G	H	D						A					N					
AN7						H							A	Y				N				G	
AN8						H							A					V		G		G	G
AN9				V		H					I		A					N					
AN10						H						D	A					N					
AN11*	A					H				N			A					G			G		

FIGURE 1: Sequences of the clones AN1−11 selected after three rounds of DNA shuffling and screening for guaiacol activity. The asterisk denotes that due to the deletion of the first base of the stop codon, AN11 has an extension of 11 amino acids (SEAFLANGSGC).

Libraries of CCP were constructed by DNA shuffling (*24*), using either Mg^{2+} or Mn^{2+} as the metal ion in the DNase I fragmentation reactions, as well as by *error-prone* PCR (*26*). The size of the libraries after transformation was usually around 5×10^4 independent clones. Sequencing of nine randomly picked clones from the libraries obtained by DNA shuffling with Mn^{2+} as the DNase I cofactor in the fragmentation reactions showed a mutation frequency of about 0.2%. The mutation frequency of the employed *error-prone* PCR protocol has been reported to be around 0.2% (*26*), and the mutation frequency for DNA shuffling using Mg^{2+} as the cofactor in the DNase I fragmentation reactions has been reported to be around 0.7% (*24*).

Screening of hCCP Libraries. Up to 10^4 hCCP clones from the prepared libraries were plated on a 14 cm diameter nitrocellulose membrane. The screening of the hCCP libraries for increased activity against guaiacol relies on the brown color of the product tetraguaiacol (*27*), i.e., the staining of colonies expressing active hCCP. Visual inspection of the membrane allowed the selection of the fastest staining colonies. Substrates other than guaiacol, which allowed staining of colonies expressing hCCP, included dianisidine, phenylendiamine, ABTS, pyrogallol, and luminol (detection by chemiluminescence). The background staining with these substrates proved to be negligible.

We conducted three successive rounds of DNA shuffling and screening for mutants with increased activity against guaiacol. In each round, roughly 10^4 mutants were screened. After the first round of shuffling, the first colonies started to stain after 8 min, whereas colonies expressing wild-type hCCP stained after 20 min (using 20 mM guaiacol and 360 μM H_2O_2). Twenty-seven mutants were selected and subjected to DNA shuffling. After the second round, the first colonies stained after 2 min and 30 mutants were selected for another round of DNA shuffling. For the third round, lower guaiacol concentrations were used (2 mM) and the first colonies stained after 5 min, whereas colonies expressing wild-type hCCP did not stain within 60 min.

Eleven clones obtained after the third round were characterized by sequencing (Figure 1). On average, each clone has about five point mutations, the lowest number being four

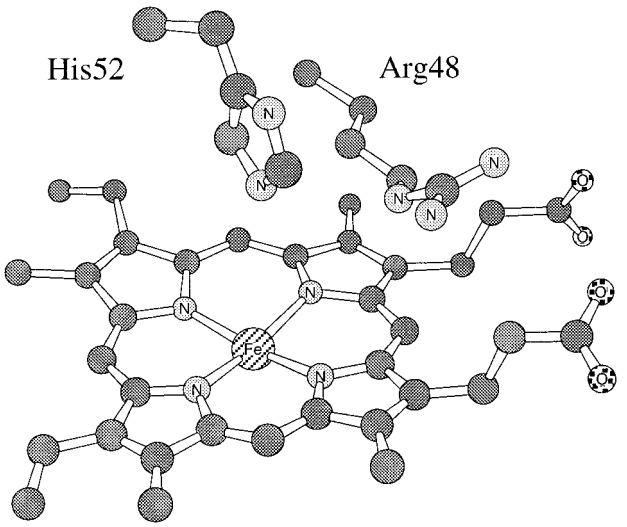

FIGURE 2: Structure of the active site of CCP showing the position of Arg48 and His52 on the distal side of the heme cofactor (*43*).

and the highest eight. In addition to six point mutations, clone AN11 also has an eleven-amino acid extension at the C-terminal end, resulting from a deletion of the first base of the stop codon TAG and a resulting frameshift. Three positions have been mutagenized in every clone: Arg48 to His, Thr180 to Ala, and Asp224 to Asn (seven times), Val (three times), or Gly (once). Arg48 is one of only nine amino acids that are completely conserved among all peroxidases of the superfamily of plant, fungal, and bacterial peroxidases (*2*). Sequence identities between individual members are as low as 16%. Arg48 is located on the distal side of the heme next to the equally conserved distal histidine (Figure 2). In CCP, Arg48 plays an important role in the stabilization of compound I (*28*, *29*), where it is believed to interact with the oxene ligand of compound I (*30*). Mutating Arg48 to Lys, Leu, or Glu decreased the stability of compound I by 2−3 orders of magnitude (*28*, *29*). Thr180 lies on the proximal side of the heme in the direct neighborhood of the propionic acids (Figure 3). Asp224 is not in direct contact with the heme or any catalytically important amino acids (Figure 3). There are also a number of mutations that appear at least twice: Asn164Asp (three times), Asn184Tyr (twice), Asp279Gly (twice), and Ser282Gly (three times). Of the mutations that have been found more than once, only Arg48His, Thr180Ala, and Asn184Tyr are in direct contact with the heme or are located in the distal cavity or the substrate access channel (Figure 3). Of the residues that have been mutated in only one of the clones of the AN1−11 series, only Val154 is in direct contact with the heme. Noteworthy also is the mutation Asp148Asn, which lies at the entrance of the substrate access channel of CCP. On the basis of structural alignment, the homologous amino acid of HRP at this position is Phe142, which is part of a distinct hydrophobic patch near the exposed heme edge and is important for the binding of small aromatic substrates (*31*). Consequently, the mutation Asp148Asn at the entrance of the substrate channel of CCP might facilitate the binding of hydrophobic substrates such as guaiacol. Of course, mutations might also arise due to neutral drift. The complexity of the structure−function relationship of CCP makes an interpretation of the selected mutations in the absence of further data very speculative.

The number of screened colonies per round (10^4) is only a small fraction of just the total number of possible double mutants for CCP (about 1.5×10^7). To test how reproducible the results of these screenings were, we constructed two independent libraries of hCCP using DNA shuffling with Mn^{2+} as the cofactor in the DNase I fragmentation reaction (library PTI) as well as *error-prone* PCR (library PTII). After one round of screening of both libraries for mutants with increased activity against guaiacol (a total of 10^4 colonies screened from each library), 10 clones that stained significantly faster than the wild type were analyzed by sequencing (Table 1). Nine of these ten clones had mutations that were also present after three rounds of DNA shuffling. Four of the ten clones possessed the mutation Arg48His, although these all were independent clones, as they all had additional (silent) mutations. One clone each also had the mutations Thr180Ala and Asp224Asn, two amino acids that are also mutagenized in each of the 11 clones obtained after three rounds of DNA shuffling. These findings show that the screening is reproducible and strongly suggest that most of the mutations found after three rounds are additive.

Characterization of Selected Mutants AN1−11. The clones AN1−11 were expressed and purified as described for wild-type hCCP. The UV spectra of the clones were all very similar and exhibited a small shift of the Soret band to a higher wavelength (409.2 nm instead of 408.2 nm for the wild type) and a slightly higher R_Z value than the spectrum of wild-type hCCP (1.25−1.5). The spectroscopic properties of the mutant AN5 are listed in Table 2. These data, in particular, the ratios A_{Soret}/A_{380} and A_{620}/A_{638} (i.e., A_{620}/A_{CT1}), suggest that AN5, as well as the other mutants AN1−11, is mostly a mixture of the penta- and hexacoordinated high-spin form (*32*). Similar results have been reported for an Arg48Glu mutant of CCP (*29*).

The activities against guaiacol, pyrogallol, cytochrome *c*, and $K_4Fe(CN)_6$ are shown in Table 3. Thus, three rounds of successive DNA shuffling and screening improved the activity of CCP up to 300-fold against small phenolic substrates, whereas in comparison, the changes in activity against the natural substrate cytochrome *c* as well as $K_4Fe(CN)_6$ were relatively small. These data indicate that the significant increases in activity against guaiacol and pyrogallol are not due to a general increase in peroxidase activity but rather specific for phenolic substrates.

The measured velocity of the guaiacol oxidation as a function of the guaiacol concentration for mutant AN10 is shown in Figure 4. Very similar results were obtained for the other mutants (AN1−11). At guaiacol concentrations of <5 mM, a rapid inactivation of the enzyme was observed, resulting in a nonlinear increase in the rate of the reaction as the guaiacol concentration is increased. The observed rate constants k_{obs} were 0.46 mM s^{-1} at 1 mM guaiacol and 1.55 mM s^{-1} at 2 mM guaiacol. The rapid inactivation of the selected mutants at low guaiacol concentrations can be rationalized by the low stability of compound I due to the Arg48His mutation (vide infra) (*28*, *29*). This instability of compound I results in a competition between its reduction by guaiacol and its endogenous reduction and inactivation of the enzyme (*33*, *34*). Second, at guaiacol concentrations of >15 mM, an inhibition of the reaction with increasing guaiacol concentrations was observed. This substrate inhibition is not observed with hCCP. However, similar effects

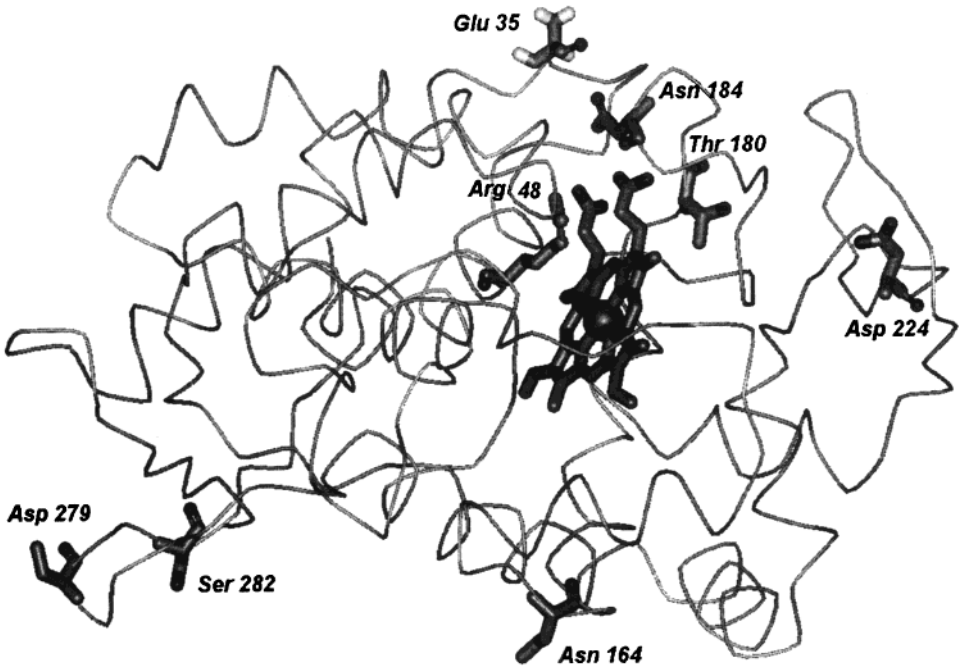

FIGURE 3: Locations of substituted residues in the structure of CCP (*43*). Only the residues that have been mutated more than once in clones AN1−11 are shown.

Table 1: Clones Isolated from Libraries PTI and PTII

clone	library	mutations[a]
PT1	PTI	Arg48His
PT2	PTI	*Asn184Ile*, Lys243Gln
PT3	PTI	Lys12Ile, *Asp224Asn*
PT4	PTI	Thr180Ala
PT5	PTI	*Arg48His*, Asp136Asn
PT6	PTI	Val47Ala, *Arg48His*
PT7	PTI	Arg48His
PT8	PTI	Ser246Gly
PT9	PTII	*Arg48His*, Phe77Tyr
PT10	PTII	Leu232Pro

[a] Mutations also found in clones AN1−11 are shown in italic; silent mutations are not listed.

have been observed for chloroperoxidase, where the binding of the substrate in the distal cavity prevents the formation of compound I (*35*). Although the low rate of turnover of the mutants at low guaiacol concentrations and the inhibition by guaiacol at high guaiacol concentrations prevent the calculation of k_{cat} and K_M values from this set of data, we nevertheless can calculate a lower limit for the specificity constant k_{cat}/K_M (calculated from the data listed in Table 3). The lower limit of k_{cat}/K_M for the most active mutant AN5 is 22.4 s^{-1} mM^{-1}, and thus at least a factor of 230 above the measured k_{cat}/K_M value for hCCP (Table 4) and only a factor of about 4 below the value of HRP (*36*).

To investigate how the selected mutations, in particular, the mutation Arg48His, affect the rate of formation of compound I and its stability, the reaction of the mutant AN5 with H_2O_2 was characterized by stopped-flow kinetics (Figure 5). To confirm the formation of compound I, a spectrum after reaction for 1.04 s was recorded using a diode array spectrometer (integration time of 14 ms). The Soret maximum is shifted to 413 nm, and two charge-transfer bands at 529 and 551 nm appear, this being consistent with the formation of compound I as an intermediate (*33*, *34*). Formation of compound I under pseudo-first-order conditions ($[H_2O_2] \gg [E]$) can be fitted to a single-exponential function. The bimolecular rate constant for the formation of compound I is $(9.3 \pm 0.3) \times 10^6$ M^{-1} s^{-1}. This is only a factor of 4 lower than the value reported for the wild-type enzyme (*37*), and thus indicates that the mutation Arg48His or any of the other mutations present in mutants AN1−11 do not significantly effect the rate of formation of compound I. These data are in agreement with the rate constants of compound I formation obtained for other Arg48 mutants (*28*, *29*). For example, the rate constant for compound I formation of the mutant Arg48Lys is only a factor of 2 less than the value of the wild type (*28*). However, the stability of compound I of AN5 is drastically reduced compared to that of wild-type CCP. The corresponding decay of the absorbance at 424 nm can best be fitted to a two-exponential function. The rate constant describing the process with the large amplitude (about 80%) has a value of 0.041 s^{-1} at 2.5 μM H_2O_2, and the process with the smaller amplitude has a value of 0.016 s^{-1} at 2.5 μM H_2O_2. Compared to that of compound I of wild-type CCP, the stability of compound I of AN5 is decreased by 3 orders of magnitude (*37*). Again, the decrease in stability of AN5 can be best explained with the mutation Arg48His.

A pH profile for the mutants AN5, AN9, and AN11 (which carry Asn, Val, and Gly at position 224, respectively) with guaiacol as a substrate was measured from pH 4.5 to 8 (Figure 6). As with wild-type hCCP, there is an increase in activity from pH 4.5 to 6.5. However, in contrast to wild-type hCCP, the mutants show a decrease in activity at pH >6.5, which is partially due to the inactivation of the enzyme under these conditions. A possible explanation is that in the selected mutants, His48, to substitute for Arg48, needs to be protonated for the stability and activity of the enzyme.

Role of the Mutation Arg48His. The most common mutation found in clones isolated from one round of screening is Arg48His. The activity of clone PT1 (Table 1), which possesses only the mutation Arg48His, is 26 s^{-1} under

Table 2: UV Spectrum of AN5 in 100 mM KP_i

	absorption maxima nm (mM^{-1} cm^{-1}); sh = shoulder							
pH	δ	Soret	CT2	β	α	CT1	A_{Soret}/A_{380}	A_{620}/A_{638}
4	~378 (55.4, sh)	409.5 (109.6)	505 (10.9)	~542 (8.2, sh)		637.5 (3.7)	1.9	0.87
6		409 (117.9)	505.5 (10.3)	~536 (9, sh)		639 (2.8)	2.1	0.79
8	~377 (41.5, sh)	409.5 (114.6)	505.5 (8.5)	~536 (8, sh)	~566 (5.1, sh)	634 (2.7)	2.5	0.88

Table 3: Apparent First-Order Rate Constants for the Oxidation of Different Substrates at pH 6 and 30 °C[a]

	guaiacol (s^{-1})	pyrogallol (s^{-1})	cytochrome *c* (s^{-1})	$K_4Fe(CN)_6$ (s^{-1})
hCCP	0.37 ± 0.01	2.37 ± 0.3	737 ± 10	262 ± 10
AN1	85.9 ± 1	237 ± 20	349 ± 20	270 ± 10
AN2	81.5 ± 1	241 ± 10	525 ± 30	365 ± 10
AN3	70.4 ± 2	172 ± 10	149 ± 50	78 ± 10
AN4	74.9 ± 1	221 ± 10	680 ± 30	284 ± 20
AN5	112.0 ± 2	260 ± 30	751 ± 30	535 ± 20
AN6	72.1 ± 2	138 ± 10	399 ± 30	96 ± 10
AN7	60.5 ± 3	181 ± 10	414 ± 10	214 ± 10
AN8	87.6 ± 2	197 ± 20	728 ± 30	440 ± 20
AN9	89.2 ± 3	266 ± 10	1102 ± 20	233 ± 10
AN10	78.3 ± 3	217 ± 20	868 ± 50	370 ± 10
AN11	73.4 ± 2	215 ± 20	109 ± 10	335 ± 20

[a] Detailed conditions for the different substrates are described in Experimental Procedures.

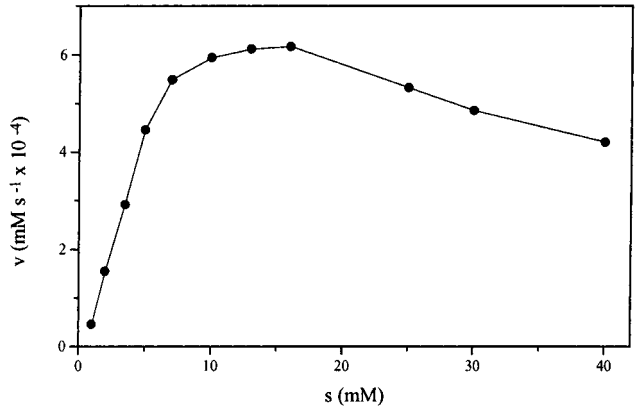

FIGURE 4: Dependence of the measured velocity in the AN10-catalyzed oxidation of guaiacol as a function of the guaiacol concentration. The reactions were assessed at 30 °C and pH 6 with 50 mM KH_2PO_4, 170 μM H_2O_2, and 5 nM AN2.

the conditions of Table 3 and thus about 70-fold higher than that of hCCP, and it possesses already about 25% of the activity of the most active clone. Furthermore, the mutant with the single mutation Arg48His exhibits faster inactivation and therefore a lower turnover with guaiacol as a substrate compared to AN5. The average turnover per enzyme before inactivation under the conditions described in Table 3 is about 1100 for PT1 and 6200 for AN5. Thus, additional mutations have increased the ratio of the rates of guaiacol oxidation relative to enzyme inactivation in the course of the directed molecular evolution of hCCP. As with mutants AN1−11, PT1 fails to form a stable compound I (data not shown). The activities of PT1 against cytochrome *c* as well as $K_4Fe(CN)_6$ have not significantly changed, the relative activities compared to wild-type hCCP under the conditions described in Table 3 being 0.5 and 0.7, respectively. These data show that the mutation Arg48His is to a large extent responsible for the increased guaiacol activity of the mutants AN1−11 as well as the observed instability of compound I.

Role of Mutations Thr180Ala and Asp224Asn. To address the role of the mutations Thr180Ala and Asp224X (X = Asn, Val, or Gly), the properties of the mutants PT3 and PT4 were examined. In addition to the mutation Asp224Asn, PT3 also carries the mutation Lys12Ile. However, as Lys12 is located on the surface of CCP and the mutation Lys12Ile was not found in any of the mutants obtained after three rounds of selection, the influence of this mutation on the properties of PT3 was neglected. Plasmids encoding the mutants PT3 and PT4 reduced the time required for staining of colonies by a factor of approximately 4. However, the activities of the purified proteins against guaiacol showed no major differences with respect to hCCP (Table 4). In both cases, the k_{cat} and K_M values for guaiacol were slightly reduced compared to those for hCCP and the specificity constant k_{cat}/K_M was within 20% of the value for hCCP. Similar tendencies were seen for the substrate $K_4Fe(CN)_6$, although the K_M of PT4 for $K_4Fe(CN)_6$ was decreased about 6-fold (Table 4). A faster staining of a colony could also be achieved by simply increasing the concentration of the holo form of hCCP in *E. coli*. To investigate if this was the case for the mutants PT3 and PT4, the enzymes were isolated by affinity purification without adding hemin after lysis of the cells. No significant variations in the amount of total protein isolated could be detected (3.88 ± 0.42 mg for wild-type hCCP, 4.17 ± 0.22 mg for PT3, and 4.23 ± 0.35 mg for PT4 per 150 mL of shake flask culture). However, in the case of PT3 and PT4, an about 2−3-fold increase in the amount of the isolated holo form was observed. Under these conditions, the percentage of the holo enzyme for wild-type hCCP was 0.86 ± 0.36%, whereas the value for PT3 was 2.6 ± 0.4% and the value for PT4 1.8 ± 0.2%. The amount of total, extractable heme in *E. coli* has been reported to be about 25 pmol mL^{-1} $OD_{600}{}^{-1}$ (*38*). The amounts of the holo form of wild-type hCCP isolated from *E. coli* Bl21(DE3) after expression are 4.3 ± 1.8 pmol mL^{-1} $OD_{600}{}^{-1}$. We therefore conclude that there is intracellular competition for heme between heme-binding host proteins and hCCP, with a significant fraction of the heme scavenged by the host proteins. Consequently, mutations that increase the affinity of the apo form of hCCP for the heme (or rather increase the rate constant for the formation of the holo form) will also be selected under the conditions used in our screening system.

Role of Asn184. Asn184 has been mutated to tyrosine in two of the 11 mutants isolated after three rounds of subsequent DNA shuffling and screening (mutants AN3 and AN7) and to isoleucine in mutant PT2, which has been obtained after one round of screening of independent libraries. Asn184 is located at the γ side of the porphyrin and its side chain amide makes a hydrogen bond to the propionic acid of the heme (distance of 2.93 Å). It is one of the amino acids that is part of the distal cavity near the substrate entrance channel in CCP, the distance between the nitrogen of the

Table 4: Kinetic Parameters of Mutants PT2−4 for the Oxidation of Various Substrates at pH 6 and 30 °C[a]

	guaiacol			$K_4Fe(CN)_6$			cytochrome *c*[b]
clone	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{rel}
hCCP	6.9 ± 1.8	71.7 ± 21.4	0.097 ± 0.003	309 ± 10	12.2 ± 0.6	25.3 ± 0.3	1 ± 0.15
PT2	16.7 ± 4.5	53 ± 17	0.316 ± 0.016	465 ± 38	13.7 ± 1.6	33.9 ± 1	1.45 ± 0.14
PT3	3.86 ± 1.18	33.2 ± 13.2	0.116 ± 0.01	316 ± 15	9.1 ± 0.7	34.7 ± 1.1	1.05 ± 0.05
PT4	2.8 ± 0.1	27.9 ± 1.3	0.1 ± 0.001	82.5 ± 1.7	2.3 ± 0.16	35.9 ± 1.8	0.32 ± 0.015

[a] Detailed conditions for the determination of the parameters are described in Experimental Procedures. [b] For clarity, data are given as relative constants compared to that of hCCP. The absolute value for the oxidation of cytochrome *c* by hCCP is given in Table 3.

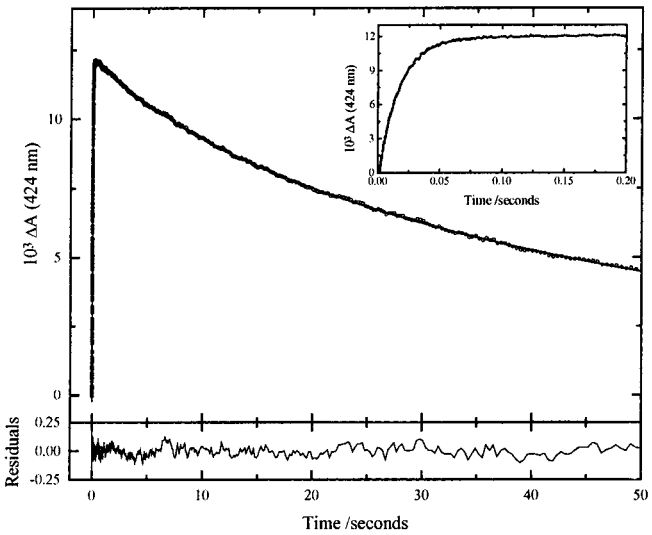

FIGURE 5: Stopped-flow trace at 424 nm observed upon mixing 0.5 μM AN5 and 5 μM H_2O_2 at pH 6 and 25 °C. The increase at 424 nm (inset) corresponds to compound I formation, and the subsequent decrease is attributed to its decomposition. The traces are the average of three experiments, and the reaction can be best fitted to a three-exponential function.

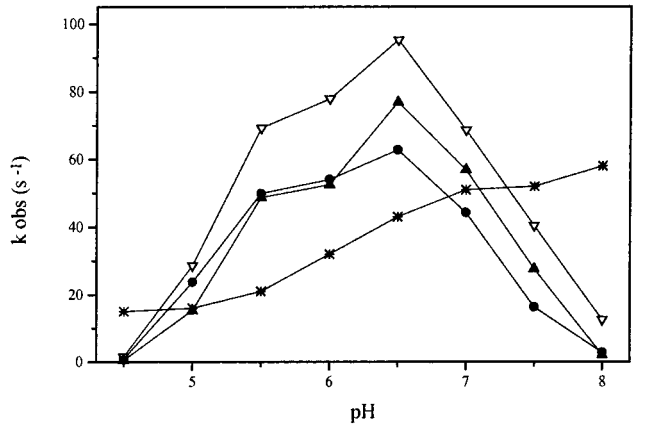

FIGURE 6: pH profile for the oxidation of guaiacol by mutants AN5 (▽), AN9 (▲), and AN11 (●) and wild-type hCCP (*). The k_{obs} values (obtained by dividing the measured velocity by the enzyme concentration) for wild-type hCCP are multiplied by a factor of 100.

side chain amide of Asn184 and the N_ϵ of Arg48 being 4.7 Å. To investigate the influence of the deletion of the hydrogen bond to the propionic acid and the introduction of a hydrophobic amino acid at this position in CCP, we characterized the kinetic properties of PT2 (Table 4). In addition to the mutation Asn184Ile, PT2 also bears the mutation Lys243Gln. However, as this mutation is located on the solvent-exposed side of helix I and the mutation Lys243Gln was not found in any of the mutants obtained after three rounds of selection, we have neglected its influence on the properties of clone PT2. The k_{cat} of PT2 is increased about 2.4-fold against guaiacol, and the K_M is lowered by a factor of about 1.4. Accordingly, the mutation Asn184Ile increased the specificity constant k_{cat}/K_M by a factor of about 3. Furthermore, the activity against the natural substrate cytochrome *c* is increased by a factor of 1.45, and the k_{cat} for $K_4Fe(CN)_6$ is increased by a factor of 1.5. These data indicate that the mutation Asn184Ile in CCP increases the general reactivity of the enzyme.

DISCUSSION

The goal of this work was to establish a system that allows for the directed molecular evolution of CCP. Subsequently, we wanted to use this system to generate mutants with increased activity against the classical peroxidase substrate guaiacol, thus shifting the specificity from a protein as a substrate toward a small organic molecule. Taking advantage of the fact that CCP can be functionally expressed in high yields in *E. coli* and that a number of peroxidase substrates yield colored, fluorescent or chemiluminescent products, we used a simple colony screen to examine up to 10^4 different mutants in a single experiment. Substrates that can be used in the screen include phenols, aromatic amines, ABTS, $K_4Fe(CN)_6$, and luminol.

Using this screen, mutants with up to 300-fold increased activity against guaiacol were isolated after three rounds of DNA shuffling and screening. The specificity toward guaiacol versus the natural substrate cytochrome *c* is increased up to 1000-fold. Under the employed screening conditions, the same mutations were repeatedly identified from independent libraries. Mutations that have been analyzed in greater detail and lead to a faster staining of the colonies can be divided into two groups: mutations that increase the intracellular concentration of active CCP and mutations that increase the peroxidase activity against the substrate guaiacol.

Mutations that increase the intracellular concentration of active hCCP but do not significantly affect the activity of the protein against guaiacol are Thr180Ala and Asp224Asn. These two mutations increase the affinity of the apo form of the peroxidase for the cofactor, as shown by a 2−3-fold higher heme content after expression and purification of the protein.

The biggest impact on the activity against guaiacol comes from the mutation Arg48His. The data for the selected mutants AN1−11 and PT1, i.e., the instability of their compound I and the rapid inactivation at low guaiacol concentrations, confirm the importance of Arg48 in the stabilization of compound I (*28*, *29*). The measured rate constant for compound I formation of mutant AN5, which is only a factor of 4 less than that of the wild type, confirms

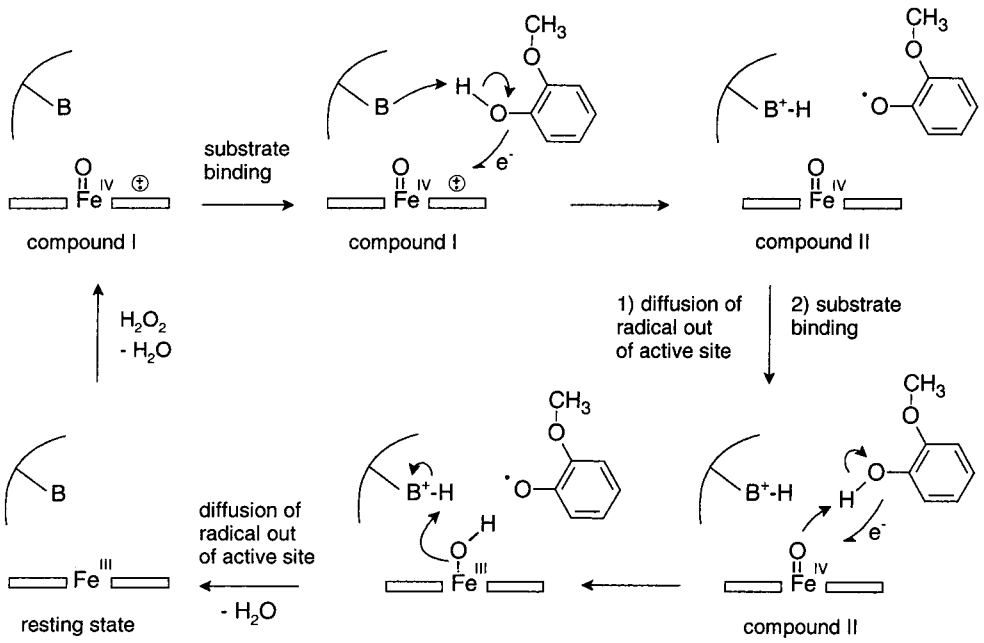

FIGURE 7: Proposed mechanism for the oxidation of phenols by HRP (*39*, *40*). B represents the distal His42 of HRP.

the observation that Arg48 does not play a critical role in the formation of compound I (*28*, *29*).

How does the mutation Arg48His then increase the activity of CCP against phenols such as guaiacol? The oxidation of phenols by HRP has been the center of intense research, and a scheme of the mechanism is shown in Figure 7 (*39*, *40*). In short, electron transfer from the substrate to the heme is accompanied by simultaneous proton transfer from the phenol to either the distal histidine (in the case of compound I) or the ferryl oxygen (in the case of compound II). A water molecule might mediate the proton transfer in both cases (*40*). Arg38 in HRP (which is equivalent to Arg48 in CCP) contributes to the binding of the substrate and its interaction with both compound I and compound II (*31*, *40*). In the ternary complex of HRP with the phenolic substrate ferulic acid and cyanide, the N_η of Arg38 forms a hydrogen bond to the phenolic oxygen of ferulic acid (*40*). This hydrogen bond is believed to assist the proton transfer to the distal histidine (reduction of compound I) or to the oxyferryl (Fe^{4+}=O) species (reduction of compound II). In a successful attempt to increase the peroxygenase activity of HRP, Ortiz de Montellano and co-workers mutated Arg38 of HRP to either Ala or His (*41*). As the peroxygenase activity is to a large extent controlled by the access of the substrate to the ferryl oxygen, these experiments also demonstrate that Arg38 in HRP plays a role in controlling the steric access to the reactive ferryl oxygen. In accordance with the proposed role of the hydrogen bond between N_η of Arg38 and the phenolic oxygen of the substrate in the oxidation of phenols (*40*), the k_{cat} values of the Arg38His and Arg38Ala mutants against guaiacol were decreased 2- and 55-fold, respectively (*41*). However, the K_M values for guaiacol for both mutants were also decreased 10-fold. It therefore can be stated that Arg38 in HRP, in addition to its role in the formation and stabilization of compound I, controls the steric access in the distal cavity.

Taking into account the mechanism of phenol oxidation and the role of the distal arginine in HRP, we propose that the mutation Arg48His in CCP increases the steric access of the phenolic substrate to both the distal His52 and the ferryl oxygen. This would facilitate the required proton transfer from the phenol to either the distal His52 or the ferryl oxygen of compound II. As the reduction of compound II is usually slower than the reduction of compound I (*39*), the facilitated proton transfer to the ferryl oxygen of compound II might be more important for the observed acceleration than the proton transfer to His52. This mechanism would be in agreement with the observed specific increase in activity against phenols, whereas the activity against $K_4Fe(CN)_6$, a substrate where no proton transfer from the substrate to the protein is required but which is also oxidized at the distal side of the heme (*6*), is not significantly affected. The relatively small changes in activity against cytochrome *c* also indicate that the mutation Arg48His does not change the general reactivity of the heme to a large extent. The hypothesis that guaiacol binds differently in the distal cavity of mutants AN1−11 compared to wild-type hCCP is also supported by the inhibition of the guaiacol oxidation at high concentrations of guaiacol. The fact that Arg48 has only been mutated to histidine could be rationalized considering that this mutation maintains a positive charge in the distal cavity and, possibly, a hydrogen bond to the ferryl oxygen. His48 would thus be capable of substituting for Arg48 in certain respects, but still would increase the steric access to the ferryl oxygen and His52. The possibility that His48 itself participates in general base catalysis appears possible but less likely, as the distal His52 in CCP is in an ideal position to act as a general base catalyst and since the measured pH profiles indicate that His48 is protonated in the active state of the peroxidase. The proposed mechanism implies that Arg48 in CCP, as the homologous Arg38 in HRP, plays an important role in controlling the access to the ferryl oxygen and the distal histidine. Considering that the distal arginine is not absolutely essential for the stabilization of compound I in HRP (*42*), or for the catalysis of its formation in CCP (*28*, *29*), but appears to play an important role in controlling the access to the active site in both proteins, one could argue that it is its role as a gatekeeper in the active site that has led to its conservation in the evolution of peroxidases.

A mutation that increases the general reactivity of CCP is Asn184Ile. This mutation, selected from the library PTI, increases the activity against guaiacol, $K_4Fe(CN)_6$, and ferrocytochrome *c*. In addition, Asn184 has been mutated to Tyr in two of the 11 clones obtained after three rounds of DNA shuffling and screening. Our data indicate that a more hydrophobic distal cavity of CCP and the deletion of the hydrogen bond to the propionic acid destabilize compounds I and II relative to the resting state and therefore increase the reactivity of the peroxidase.

Furthermore, there are a number of additional mutations in the selected mutants AN1−11, which are scattered over the structure of CCP. The role of these mutations in the directed molecular evolution of CCP is not obvious. Additional experiments and structural information are needed to address these questions as well as to validate the hypotheses on the discussed mutations. The difficulties in understanding the effects of some of the selected mutations demonstrate the complexity of the structure−function relationship of CCP.

In summary, our experiments on the directed molecular evolution of CCP have shown that this is a powerful method for rapidly generating CCP mutants with interesting properties and improving our understanding of the structure−function relationship of this enzyme. The selected mutations leading to an increase in activity against guaiacol, in particular, Arg48His, would not have been obvious candidates in the rational design of CCP mutants with increased activity against guaiacol and thus show the strength of a combinatorial approach. Selections for CCP mutants with increased peroxidase activity against different substrates should help us to identify and clarify the role of other catalytically important amino acids. Furthermore, the screen will allow us to use techniques such as saturation mutagenesis to address the role of specific amino acids in catalysis and protein folding of CCP.

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