Biochemical and Biophysical Research Communications **286,** 126–132 (2001)
doi:10.1006/bbrc.2001.5366, available online at http://www.idealibrary.com on IDEAL®

Changing the Substrate Specificity of Cytochrome *c* Peroxidase Using Directed Evolution[1]

André Iffland, Susanne Gendreizig, Petra Tafelmeyer, and Kai Johnsson[2]
Institut de Chimie Organique, Université de Lausanne, CH-1015 Lausanne, Switzerland

Received July 5, 2001

Cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* was subjected to directed molecular evolution to generate mutants with increased activity against 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Using a combination of DNA shuffling and saturation mutagenesis, mutants were isolated which possessed more than 20-fold increased activity against ABTS and a 70-fold increased specificity toward ABTS compared to the natural substrate. In contrast, activities against another small organic molecule, guaiacol, were not significantly affected. Mutations at residues Asp224 and Asp217 were responsible for this increase in activity. These two residues are located on the surface of the protein and not in the direct vicinity of the distal cavity of the peroxidase, where small organic substrates are believed to be oxidized. Mutations at position Asp224 also lead to an increased amount of the active holoenzyme expressed in *Escherichia coli,* favoring the selection of these mutants in the employed colony screen. Possible explanations for the effect of the mutations on the *in vitro* activity of CCP as well as the increased amount of holoenzyme are discussed.

Key Words: **cytochrome *c* peroxidase; directed evolution; substrate specificity.**

Cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* is an iron protoporphyrin IX-containing peroxidase that catalyzes the oxidation of ferrocytochrome *c* by H_2O_2 (1). CCP has been shown to be an ideal system for studying the protein-cofactor interactions that control the chemistry of heme-containing enzymes. Using rational design as well as evolutionary approaches, properties such as substrate specificity and the type of reaction catalyzed by CCP have been modified, yielding important insights into the structure-function relationship of heme-containing proteins in general (2–9).

The first step in the mechanism of CCP is the reaction of the resting state of the enzyme (Fe^{3+}CCP) with H_2O_2 to form an oxyferryl (Fe^{4+}═O) species and an indolyl cation radical at position Trp191 [(Fe^{4+}═O)CCP$^{\cdot+}$], the so-called compound I (1, 10). Compound I of CCP then oxidizes one molecule of ferrocytochrome *c* (Fe^{2+}Cc) to ferricytochrome *c* (Fe^{3+}Cc), thereby being converted to an oxyferryl species [(Fe^{4+}═O)CCP], which is called compound II. In the crystal structure of the CCP–cytochrome *c* complex the two hemes are more than 17 Å apart and the electron transfer is believed to occur via the indolyl cation radical at the proximal side of the heme of CCP (11). Compound II subsequently oxidizes another molecule of ferrocytochrome *c*, converting CCP back into its resting state.

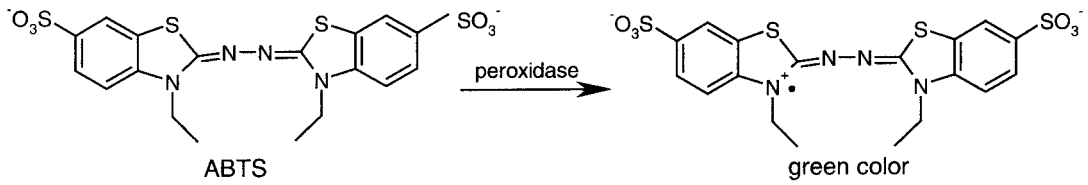
CCP is a member of the superfamily of bacterial, plant and fungal peroxidases. Other members of this superfamily, including horseradish peroxidase (HRP), oxidize small organic molecules, such as guaiacol and ascorbate, or metal ions such as Mn^{2+} (12). In general, oxidation of small molecules is believed to occur in or at the entrance of the distal cavity at the exposed δ-edge of the heme of the peroxidase (13). However, it has been recently shown that a number of peroxidases of this superfamily possess binding sites for their substrates on different parts of the protein surface of the peroxidase and not in the distal cavity. Examples are ascorbate peroxidase, lignin peroxidase and Mn^{2+} peroxidase (14–16). It is remarkable that within this superfamily of enzymes the locations of the substrate binding sites are not conserved. The reasons for this unusual diversity are unknown.

In addition to its natural substrate, CCP can also oxidize small organic molecules such as guaiacol, although with rates orders of magnitude below HRP (13). Using directed evolution, we have previously isolated CCP-mutants with up to 300-fold increased activity against guaiacol (9). Responsible for the dramatic increase in activity is the mutation Arg48His in the distal cavity. The mutation increases the steric access

[1] Funding of this work was provided by the Swiss Science Foundation, the Volkswagen-Stiftung (I/72 578), and the European Community (Grant BMH4-CT97-2277).

[2] To whom correspondence and reprint requests should be addressed. Fax: 41-21-692 3965. E-mail: kai.johnsson@ico.unil.ch.

0006-291X/01 $35.00



SCHEME 1. Oxidation of ABTS by peroxidases.

to the oxyferryl species and facilitates the for the oxidation of phenols required simultaneous electron and proton transfer from the substrate to the peroxidase (17). Activities against non-phenolic substrates were not significantly affected. The question then arose which mutations would increase the activity against substrates that do not require simultaneous proton and electron transfer, such as the classical peroxidase substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Scheme 1). Here we report the directed evolution of CCP toward an increased activity against ABTS. In the course of these experiments, mutants were isolated with a more than 20-fold increased activity against this substrate. Mutations of residues outside the distal cavity of CCP (Asp217 and Asp224) were responsible for the increase in activity.

MATERIALS AND METHODS

Reagents. Chemicals were purchased from Fluka AG. Enzymes for recombinant DNA work were purchased from MBI Fermentas or New England Biolabs. DNaseI (from bovine pancreas, cell culture grade) was purchased from Roche Molecular Biochemicals. Cytochrome *c* (from horse heart) was purchased from Fluka AG. For the affinity purification Ni–NTA–agarose (Qiagen) was used. Libraries were plated on Hybond-C supermembranes (Amersham Life Sciences).

Oligonucleotides. The following primers were used: AI5851, 5′-CTAAGAGCGGCTACATGATGCTGC-3′; AI5852, 5′-GCATCATGT-AGCCGCTCTTAGAVNNCCACTGTTCGTTGTTCGCVNNGTTCTT-TTCCAATTTC-3′; AI3461, 5′-CGATATAGGCGCCAGCAACC-3′; AI3462, 5′-AAGCTTTAATGCGGTAGTTTATCACAGT-3′; N: A, T, G, C; V: A, G, C.

Construction and screening of libraries and expression of selected clones. For the construction and screening of CCP libraries as well as the expression of individual mutants the previously described plasmid phCCP was used (9). In this plasmid, the CCP gene is downstream of the T7-promoter, possesses an N-terminal 6×His tag (hCCP) and is placed between two different *Sfi*I sites. DNA shuffling for the preparation of the initial libraries and for the shuffling of selected mutants was performed following the protocol of Stemmer *et al.* but using Mn^{2+} as the cofactor for DNase I (18). The size of the resulting libraries was around 5×10^4. For the saturation mutagenesis at positions 217 and 224 SOE PCR using primers AI5851, AI5852, AI3461 and AI3462 was performed (19). Pfu DNA polymerase was used in these experiments. The size of the library after electroporation into BL21(DE3) was 2×10^4 independent clones.

For the screening of the libraries up to 5000 colonies were directly spread on a nitrocellulose membrane (14-cm diameter) and the membrane was incubated on a LB agar plate (100 μg/ml amp) for 16 h at 27°C. The size of the colonies was below 1 mm in diameter. For induction of protein expression, the membrane was then transferred on a new LB agar plate (1 mM IPTG, 100 μg/ml amp) and 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 soaked with 50 mM KH_2PO_4, pH 6, 2 mM ABTS, 0.035% H_2O_2 and kept at 4°C during the screening. The filter was visually inspected for colonies turning green and the fastest staining colonies were manually picked and amplified overnight in LB medium (100 μg/ml amp). The wild-type stained after approximately 20 min under these conditions. The libraries prepared by saturation mutagenesis at residues 217 and 224 were screened at 5 mM ABTS at room temperature.

CCP mutants were expressed and purified as previously described (9). Heme content was determined by the pyridine hemochromogen assay (20). Protein concentrations were measured by the Bradford assay (21). Purity was checked by SDS–gel electrophoresis and was greater than 95%.

Kinetic measurements. All reported data are the average of at least three independent measurements. ABTS measurements were taken at 30°C, pH 6, 50 mM KH_2PO_4, 0.1 mg/ml BSA, 0.25 mM ABTS, and 250 μM H_2O_2 using a microtiter plate reader (Spectra Max 340, Molecular Devices). The enzyme concentrations were 10 nM for hCCP and 2.5 nM for the mutants. The absorbance was recorded at 414 nm (ε = 36 mM^{-1} cm^{-1}). All other measurements were taken on a UV spectrophotometer (Lambda 10, Perkin–Elmer) at 30°C. Oxidation of guaiacol was measured at pH 6, 50 mM KH_2PO_4, 170 μM H_2O_2, 5 mM guaiacol and enzyme concentrations of 500 nM by monitoring the absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) (13). Oxidation of horse heart ferrocytochrome *c* was measured at pH 6, 50 mM KH_2PO_4, 180 μM H_2O_2, 40 μM ferrocytochrome *c* and enzyme concentrations of 500 pM by monitoring the absorbance at 550 nm ($\Delta\varepsilon$ = 18.5 mM^{-1} cm^{-1}) (2). Oxidation of $K_4Fe(CN)_6$ was measured at pH 6, 100 mM KH_2PO_4, 600 μM H_2O_2, 17 mM $K_4Fe(CN)_6$ and enzyme concentrations of 75 nM by monitoring the absorbance at 420 nm (ε = 1 mM^{-1} cm^{-1}) (13).

RESULTS

The library for the first round of screening was prepared by DNA shuffling of the CCP wild-type gene. Sequencing of nine randomly picked clones of the so prepared library showed that the members of the library possess on average two random point mutations per gene. 10^4 CCP clones of this library were then screened for higher activity against ABTS. The screening relies on the functional expression of CCP in *E. coli* and a simple colony screen. Colonies expressing active CCP turn green in the presence of ABTS and H_2O_2 due to the intense color of the product of the ABTS oxidation (Scheme 1). Background staining of *E. coli* BL21(DE3) not expressing CCP was negligible. After the first round of screening, 19 clones that stained faster than wild-type were isolated and subjected to

TABLE 1

Sequences of the Clones K2.1–K2.4

Residue No.	39	60	88	93	184	217	224	292
CCP	Y	H	G	E	N	D	D	Q
K2.1			S	G		G	V	
K2.2						G	Y	L
K2.3							Y	
K2.4	H	R			D	G	Y	

DNA shuffling. Clones (10^4) of the resulting library were used in a subsequent round of screening. Four of the fastest staining colonies were amplified and the corresponding CCP genes were sequenced (Table 1). Asp224 is mutated in each of the selected clones to either Tyr (3×) or Val (1×). Asp217 is mutated in three of the selected clones to Gly. The locations of the mutated residues in the wild type structure of CCP are shown in Fig. 1 (22).

Asp224 is in the middle strand of a short β-sheet in the C-terminal domain of the peroxidase (Fig. 2). In the neighboring strand of this β-sheet, adjacent to Asp224, is Met230. Met230 is one of the residues in direct contact with Trp191, the radical cation of compound I. The distances between the Cγ of Asp224 and the Cβ of Met230 and the Cγ of Trp191 are 3.37 and 9.50 Å, respectively. Asp217 is located in a turn between the first and the second strand of the β-sheet and its side chain points toward Asp224 (Fig. 2). The distance between the two Cγs is 10 Å. Neither Asp217 nor Asp224 are part of the residues that form the entrance channel to the distal cavity.

To measure the activity of the selected mutants *in vitro*, the proteins K2.1–K2.4 were expressed and purified using a 6×His affinity tag. The activities of the mutant peroxidases and wild-type (hCCP) against ABTS, the natural substrate cytochrome *c*, and two other peroxidase substrates, guaiacol and $K_4Fe(CN)_6$, are listed in Table 2. The activity against ABTS increased about 18-fold (K2.2), whereas the activities against the other substrates were much less affected. All four selected mutants possessed significantly higher activity against ABTS compared to wild-type hCCP (at least 9-fold), demonstrating the efficiency of the screen.

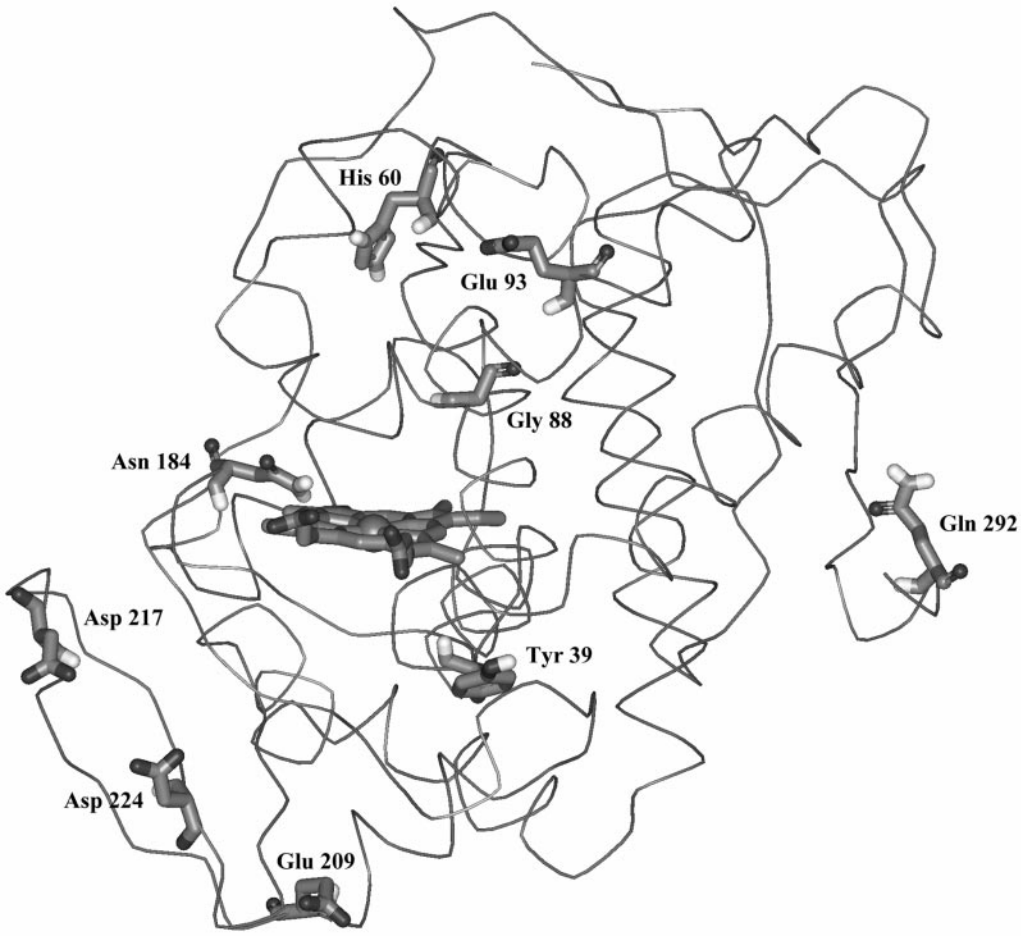


FIG. 1. Locations of mutated residues in the structure of CCP (22).

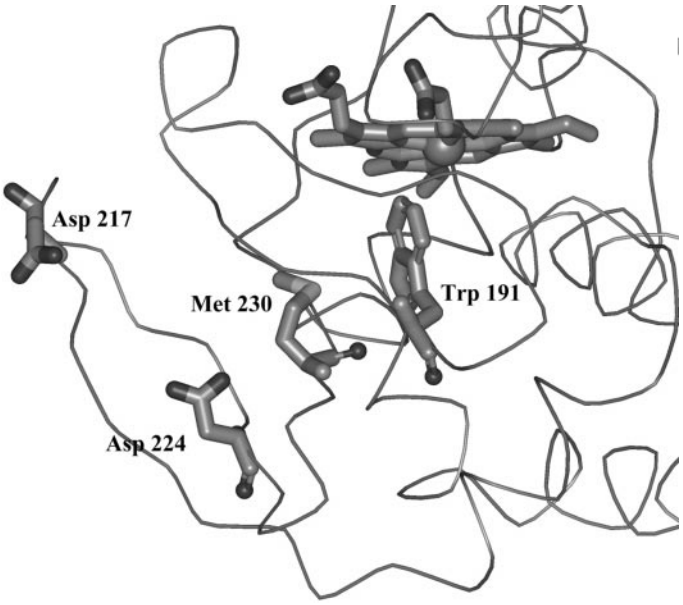


FIG. 2. Locations of residues Asp217, Asp224, Met230, and Trp191 in the structure of wild-type CCP (22).

TABLE 2

Kinetic Parameters of the Selected Mutants for the Oxidation of Various Substrates at pH 6, 30°C[a]

	ABTS k_{obs} [s^{-1}]	$K_4Fe(CN)_6$ k_{obs} [s^{-1}]	Guaiacol k_{obs} [s^{-1}]	Cytochrome *c* k_{obs} [s^{-1}]
CCP	2.4 ± 0.2	212 ± 2	0.35 ± 0.03	676 ± 14
K2.1	21 ± 1	208 ± 6	0.58 ± 0.04	299 ± 54
K2.2	44 ± 1	204 ± 11	0.33 ± 0.03	319 ± 31
K2.3	24 ± 2	165 ± 9	0.36 ± 0.01	340 ± 15
K2.4	31 ± 4	187 ± 6	0.80 ± 0.05	158 ± 2
AE1	20 ± 1	172 ± 4	0.37 ± 0.03	268 ± 10
AE2	32 ± 1	278 ± 4	0.53 ± 0.12	505 ± 21
AE3	10 ± 1	150 ± 5	0.32 ± 0.01	324 ± 10
AE4	9 ± 1	185 ± 5	0.37 ± 0.01	383 ± 15
AE5	55 ± 4	191 ± 3	0.31 ± 0.01	228 ± 19
AE7	15 ± 2	246 ± 2	0.23 ± 0.01	271 ± 25
AE8	21 ± 2	556 ± 36	0.58 ± 0.02	491 ± 20

[a] Detailed conditions for the determination of the parameters are listed under Materials and Methods.

The mutations Asp224 to Asn, Val, or Gly have been isolated in previous selections for CCP mutants with increased activity against guaiacol (9). It has been shown that the mutation Asp224Asn increased the amount of the active holoenzyme of CCP isolated after expression two- to three-fold, but did not significantly affect the activity against guaiacol. After overexpression in *E. coli,* hCCP is mostly present as the inactive apoenzyme (9). To test if the mutation Asp224Tyr also increased the concentration of active holoenzyme, we purified the mutant K2.3 without adding additional heme after cell lysis and determined both the amount of total protein and bound heme isolated. The amount of total CCP isolated was not significantly different from wild-type (1.9 mg for K2.3 and 1.8 mg for hCCP per 150 ml of shake-flask culture), whereas the percentage of the holoenzyme increased about 3-fold [(1.7 ± 0.2)% for K2.3 and (0.6 ± 0.2)% for hCCP]. These measurements and the measured activities against ABTS show that mutations at position 224 were selected because they not only increase the activity against ABTS but increase also the amount of active peroxidase inside the bacterium.

Asn184 is another amino acid that has been mutated in the screenings for increased activity against ABTS (Asn184Asp in K2.4) as well as in screenings for increased activity against guaiacol (Asn184Tyr, Asn184Ile). The corresponding amino acid in ascorbate peroxidase is Arg172, which has been shown to be important for ascorbate binding and oxidation (15). In CCP, the side chain amide of Asn184 forms a hydrogen bond to one of the propionic acids of the heme. The mutation Asn184Ile has been shown to increase the general reactivity of the enzyme (9) and K2.4 possesses also an increased activity against guaiacol. However, due to the presence of four other mutations in K2.4, it is not possible to unambiguously assign this increase in activity to the mutation Asn184Asp.

The data show that mutations at the positions 217 and 224 did affect the activity against ABTS *in vitro* but the experiments did not necessarily identify the most active mutations at these positions. Random point mutagenesis strategies heavily favor those mutations that are accessible by single base mutations per codon (23). We thus performed saturation mutagenesis at these positions, cloning an oligonucleotide into the CCP gene where the codons of Asp217 and Asp224 had been randomized. About 10^4 clones of the resulting library were screened for mutants with higher activity against ABTS, so that each of the 400 possible double mutants should have been screened at least once. 8 clones that stained significantly faster than wild-type were sequenced (clones AE1-8, Table 3). Seven of the

TABLE 3

Clones Selected after Saturation Mutagenesis at Positions D217 and D224

Clone	217	224
CCP	D	D
AE1	S	V
AE2	H	V
AE3	F	V
AE4	A	V
AE5	P	Y
AE6	P	Y
AE7[a]	P	L
AE8	R	N

[a] AE7 also carried the mutation E209D.

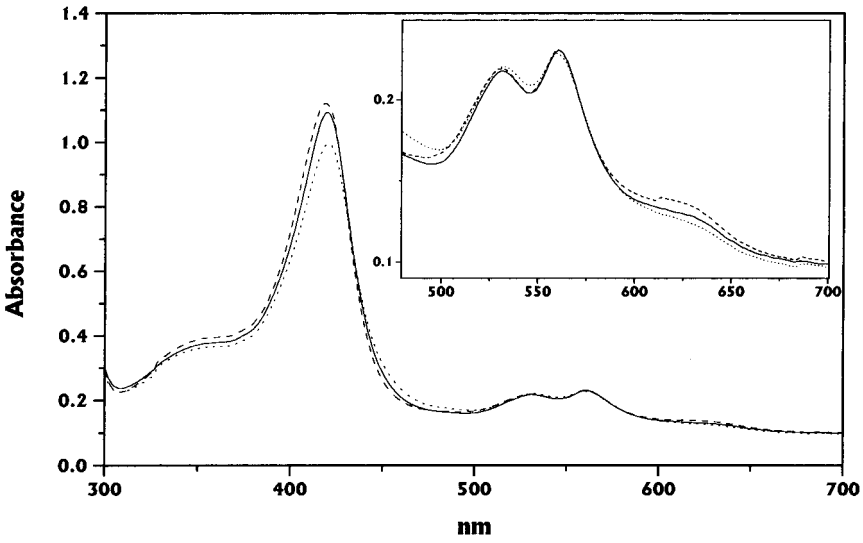


FIG. 3. UV spectra of compound I of AE5 (—), AE8 (- - -), and wild-type hCCP (– – –).

eight clones possessed hydrophobic amino acids at position 224. At position 217 either hydrophobic, neutral or positively charged amino acids were found. No negative charge at either position was isolated, the probability for this event is 17%. The combination Asp217Pro, Asp224Tyr was found twice. The fact that six of the eight sequenced clones possess either Val or Tyr at position 224 confirms the data from the random mutagenesis experiments.

The mutants AE1-8 were expressed, purified and their activities against ABTS, guaiacol, cytochrome *c* and $K_4Fe(CN)_6$ measured. The activity against ABTS increased more than 20-fold (k_{obs} for AE5) compared to wild-type, whereas the changes in activity against the other substrates were much less significant. In general, mutations at position 224 seem to have a larger effect on the activity against ABTS than mutations at position 217. Mutating Asp224 to Tyr increases the activity about 10-fold (K2.3). Introducing then the additional mutation Asp217Pro (AE5) increases the activity against ABTS by an additional factor of 2. The high variability of mutations at position 217 in the saturation mutagenesis experiments also indicates that mutations at position 224 are more important for the selection of clones than mutations at position 217.

The UV spectra of the selected mutants displayed no significant differences to wild-type hCCP. The spectra of compound I for AE5, AE8, and wild-type are shown in Fig. 3. These data indicate that the mutants possess heme-binding sites that are similar to wild-type and that they also form a compound I where the radical cation is located at Trp191. Furthermore, the stability of compound I of AE5 and AE8 compared to wild-type hCCP was not significantly affected, as judged by following the changes in the UV spectra of compound I over a period of 30 min.

DISCUSSION

The goal of this work was to change the substrate specificity of CCP from the protein cytochrome *c* toward the classical peroxidase substrate ABTS. Using a simple colony screen and a combination of random point mutagenesis and saturation mutagenesis, mutants were isolated that possessed a more than 20-fold higher activity against ABTS and a 70-fold increased specificity toward ABTS relative to the natural substrate. In our experiments, the selection of mutants relies on the staining of colonies resulting from expression of active CCP inside *E. coli* and mutations that either increase the concentration of the active holoenzyme or increase the activity against ABTS will be selected. Consequently, the selection of mutations, i.e., Asp224Tyr, that increase both the activity of the peroxidase against ABTS as well as the concentration of the active holoenzyme in *E. coli* are favored in the screening at the expense of those who only affect the peroxidase activity.

To explain how the mutations at position Asp224 can both increase the amount of the holoenzyme of the peroxidase in *E. coli* as well as enhance the activity against ABTS, we propose two different scenarios. In the first, mutations at Asp224 increase the rate constant k_{on} for the binding of the cofactor by the apoenzyme of CCP and allow the peroxidase to compete more efficiently for the heme cofactor with endogenous heme-binding proteins from *E. coli.* Mutations that affect the binding of the heme cofactor could also affect the binding of a substrate in or near the distal cavity.

Alternatively, the mutations both increase the activity against an unknown substrate that is present in *E. coli* as well as ABTS. This could protect the holoenzyme against inactivation by hydrogen peroxide, which

is generated during aerobic growth of the bacterium (24). CCP reacts with a rate constant of 3×10^7 s^{-1} M^{-1} with H_2O_2 to form compound I and slowly decomposes irreversibly in the absence of a substrate it can oxidize (25). Consequently, mutations that increase the activity against a substrate present in *E. coli* could increase the concentration of the active holoenzyme and might of course also increase the activity against ABTS. Taking into account the observed flexibility concerning the location of the substrate binding sites in peroxidases, one could imagine that mutations at the positions 217 and 224 facilitate the binding and the oxidation of ABTS or another substrate in this surface area of the protein, with electron transfer occurring via Trp191. Asp224 has been previously suggested to be part of a second binding site for cytochrome *c* which displays high activity in electron transfer from cytochrome *c* to CCP (26). The oxidation of ABTS on the surface of the protein would also explain why mutations at position Asp217 and Asp224 do not significantly affect the activity against guaiacol, which is known to be oxidized in the distal cavity (13). The activity for the most active mutant AE5 (Asp217Pro, Asp224Tyr) is increased 23-fold, whereas the activity against guaiacol is within 20% of the activity of wild-type hCCP. In addition, mutations in the distal cavity that affect the activity against guaiacol (Arg48His) do not significantly affect the activity against ABTS (27).

Although our data do not allow distinguishing between the different hypotheses, they point to the question what in the (directed) evolution of peroxidases determines where the substrate binding site for a small substrate is located. In the oxidation of phenols, the required loss of a proton from the substrate is coupled to the required proton uptake of the activated peroxidase: the phenol is bound in the distal cavity and the electron transfer from the substrate is accompanied by simultaneous proton transfer to either the distal base, in the case of compound I, or the oxyferryl species, in the case of compound II (17). In contrast, if the oxidation of the natural substrate does not require proton abstraction, there is no obvious advantage for substrate binding in or at the entrance of the distal cavity. Accordingly, specific substrate binding sites for ascorbate peroxidase, lignin peroxidase and Mn^{2+} peroxidase are located on various parts of the surface of the proteins (14–16). The results of the directed evolution experiments with CCP for mutants with increased activity against guaiacol and ABTS would follow this trend. Clearly, rationalizing the observed variability in the location of the substrate binding sites is becoming a central question in the research on peroxidases.

REFERENCES

1. Bosshard, H. R., Anni, H., and Yonetani, T. (1991) *in* Peroxidases in Chemistry and Biology (Everse, I., Everse, K. E., and Grisham, M. B., Eds.), Vol. 2, pp. 52–78, CRC Press, Boca Raton.
2. Wilcox, S. K., Jensen, G. M., Fitzgerald, M. M., McRee, D. M., and Goodin, D. B. (1996) Altering substrate specificity at the heme edge of cytochrome c peroxidase. *Biochemistry* **35,** 4858–4866.
3. Roe, J. A., and Goodin, D. B. (1993) Enhanced oxidation of aniline derivatives by two mutants of cytochrome c peroxidase at tryptophan 51. *J. Biol. Chem.* **268,** 20037–20045.
4. Bonagura, C. A., Sundaramoorthy, M., Pappa, H. S., Patterson, W. R., and Poulus, T. L. (1996) An engineered cation site in cytochrome c peroxidase alters the reactivity of the redox active tryptophan. *Biochemistry* **35,** 6107–6115.
5. Bonagura, C. A., Bhaskar, B., Sundaramoorthy, M., and Poulos, T. L. (1999) Conversion of an engineered potassium-binding site into a calcium-selective site in cytochrome c peroxidase. *J. Biol. Chem.* **274,** 37827–37833.
6. Yeung, B. K., Wang, X., Sigman, J. A., Petillo, P. A., and Lu, Y. (1997) Construction and characterization of a manganese-binding site in cytochrome c peroxidase: Toward a novel manganese peroxidase. *Chem. Biol.* **4,** 215–221.
7. Wilcox, S. K., Putnam, C. D., Sastry, M., Blankenship, J., Chazin, W. J., McRee, D. E., and Goodin, D. B. (1998) Rational design of a functional metalloenzyme: Introduction of a site for manganese binding and oxidation into a heme peroxidase. *Biochemistry* **37,** 16853–16862.
8. Hirst, J., and Goodin, D. B. (2000) Unusual oxidative chemistry of *N*(omega)-hydroxyarginine and *N*-hydroxyguanidine catalyzed at an engineered cavity in a heme peroxidase. *J. Biol. Chem.* **275,** 8582–8591.
9. Iffland, A., Tafelmeyer, P., Saudan, C., and Johnsson, K. (2000) Directed molecular evolution of cytochrome c peroxidase. *Biochemistry* **39,** 10790–10798.
10. Sivaraja, M., Goodin, D. B., Smith, M., and Hoffman, B. M. (1989) Identification by ENDOR of Trp191 as the free-radical site in cytochrome c peroxidase compound ES. *Science* **245,** 738–740.
11. Pelletier, H., and Kraut, J. (1992) Crystal structure of a complex between electron transfer partners, cytochrome c peroxidase and cytochrome c. *Science* **258,** 1748–1755.
12. Welinder, K. G. (1992) Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* **2,** 388–393.
13. DePillis, G. D., Sishta, B. P., Mauk, A. G., and Ortiz de Montellano, P. R. (1991) Small substrates and cytochrome c are oxidized at different sites of cytochrome c peroxidase. *J. Biol. Chem.* **266,** 19334–19341.
14. Doyle, W. A., Blodig, W., Veitch, N. C., Piontek, K., and Smith, A. T. (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. *Biochemistry* **37,** 15097–15105.
15. Bursey, E. H., and Poulos, T. L. (2000) Two substrate binding sites in ascorbate peroxidase: The role of arginine 172. *Biochemistry* **39,** 7374–7379.
16. Kishi, K., Kusters-van Someren, M., Mayfield, M. B., Sun, J., Loehr, T. M., and Gold, M. H. (1996) Characterization of manganese(II) binding site mutants of manganese peroxidase. *Biochemistry* **35,** 8986–8994.
17. Henriksen, A., Smith, A. T., and Gajhede, M. (1999) The structures of the horseradish peroxidase C–ferulic acid complex and the ternary complex with cyanide suggest how peroxidases oxidize small phenolic substrates. *J. Biol. Chem.* **274,** 35005–35011.

18. Stemmer, W. P. (1994) Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370,** 389–391.
19. Vallejo, A. N., Pogulis, R. J., and Pease, L. R. (1995) *in* PCR Primer, a Laboratory Manual (Dieffenbach, C. W., and Dveksler, G. S., Eds.), pp. 603–612, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. Nicola, N. A., Minasian, E., Appleby, C. A., and Leach, S. J. (1975) Circular dichroism studies of myoglobin and leghemoglobin. *Biochemistry* **14,** 5141–5149.
21. Bradford, M. M. (1977) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **83,** 346–356.
22. Finzel, B. C., Poulos, T. L., and Kraut, J. (1984) Crystal structure of yeast cytochrome c peroxidase refined at 1.7-A resolution. *J. Biol. Chem.* **259,** 13027–13036.
23. Miyazaki, K., and Arnold, F. H. (1999) Exploring nonnatural evolutionary pathways by saturation mutagenesis: Rapid improvement of protein function. *J. Mol. Evol.* **49,** 716–720.
24. Gonzalez-Flecha, B., and Demple, B. (1997) Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli. J. Bacteriol.* **179,** 382–388.
25. Erman, J. E., Vitello, L. B., Miller, M. A., and Kraut, J. (1992) Active-site mutations in cytochrome c peroxidase: A critical role for histidine-52 in the rate of formation of compound I. *J. Am. Chem. Soc.* **114,** 6592–6593.
26. Leesch, V. W., Bujons, J., Mauk, A. G., and Hoffman, B. M. (2000) Cytochrome c peroxidase–cytochrome c complex: Locating the second binding domain on cytochrome c peroxidase with site-directed mutagenesis. *Biochemistry* **39,** 10132–10139.
27. Iffland, A. Unpublished data.