Structural and Mutagenesis Studies on the Cytochrome c Peroxidase from *Rhodobacter capsulatus* Provide New Insights into Structure-Function Relationships of Bacterial Di-heme Peroxidases

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Cytochrome c peroxidases (CCP) play a key role in cellular detoxification by catalyzing the reduction of hydrogen peroxide to water. The di-heme CCP from *Rhodobacter capsulatus* is the fastest enzyme (1060 s⁻¹), when tested with its physiological cytochrome c substrate, among all di-heme CCPs characterized to date and has, therefore, been an attractive target to investigate structure-function relationships for this family of enzymes. Here, we combine for the first time structural studies with site-directed mutagenesis and spectroscopic studies of the mutant enzymes to investigate the roles of amino acid residues that have previously been suggested to be important for activity. The crystal structure of *R. capsulatus* at 2.7 Å in the fully oxidized state confirms the overall molecular scaffold seen in other di-heme CCPs but further reveals that a segment of about 10 amino acids near the peroxide binding site is disordered in all four molecules in the asymmetric unit of the crystal. Structural and sequence comparisons with other structurally characterized CCPs suggest that flexibility in this part of the molecular scaffold is an inherent molecular property of the *R. capsulatus* CCP and of CCPs in general and that it correlates with the levels of activity seen in CCPs characterized, thus, far. Mutagenesis studies support the spin switch model and the roles that Met-118, Glu-117, and Trp-97 play in this model. Our results help to clarify a number of aspects of the debate on structure-function relationships in this family of bacterial CCPs and set the stage for future studies.

Cytochrome c peroxidases (CCP) are found in yeast and bacteria and likely function to protect the organism against the accumulation of toxic peroxides. Cytochrome c peroxidase from *Rhodobacter capsulatus* (Rc) is a bacterial CCP (BCCP) that is located in the periplasm (1, 2). It binds two heme groups covalently and utilizes the two cofactors as plat-forms to catalyze the two-electron reduction of hydrogen peroxide to water by a one-electron donor such as ferrocyanochrome c.

2 Cyt c (Fe²⁺) + H₂O₂

+ 2 H⁺ → 2 Cyt c (Fe³⁺) + 2 H₂O  (Eq 1)

The overall reaction for the bacterial di-heme peroxidases is similar to that in yeast and horseradish peroxidases, but the catalytic mechanism is significantly different. Eukaryotic cytochrome peroxidases possess only one, noncovalently bound heme b group, and since the reduction of peroxides requires two electrons, the reaction generates an oxy-ferryl center on the heme and a cation radical on Trp-191 or on the porphyrin ring of the heme. The second heme group in the BCCPs removes the need for cation radical formation. With the exception of the *Nitrosomonas europaea* enzyme (3), BCCPs are only active in the mixed valence state in which the high potential, electron transferring heme is in the reduced state and the peroxidatic, low potential heme is in the Fe(III) state (4–7). Upon reduction of hydrogen peroxide, one electron is abstracted from the reduced high potential heme, which becomes reoxidized; this requires intramolecular electron transfer from the high to the low potential heme. The second electron is abstracted from the peroxidatic low potential heme group with the formation of an oxy-ferryl center. The high potential heme, with a midpoint potential of +270 mV, therefore, functions as the electron transferring heme, whereas the low potential heme has a midpoint potential between −190 and −310 mV (7) and is the peroxidatic center. Structures of the bacterial cytochrome c peroxidases isolated from *Pseudomonas aeruginosa* (Pa) (8), *N. europaea* (9), and *Pseudomonas nautica* (Pn) (10) have already been determined. The structure of the *P. aeruginosa* enzyme is that of the completely oxidized, inactive enzyme with bound calcium. That from *N. europaea* is also of the completely oxidized enzyme, but the peroxidase retains activity in this state. Recently, two distinct structural snapshots of CCP from *P. nautica* became available as the result of the serendipitous reduction of the C-terminal heme group by synchrotron X-rays used for the structural studies (10). The inactive form did not contain Ca²⁺ and was captured in a closed conformation (IN-form) where the N-terminal peroxidatic heme was coordinated by six ligands, thus blocking the peroxidatic reaction from taking place. On the other hand, the active form does bind Ca²⁺ and exhibits an open conformation (OUT-form) with release of the distal histidine ligand, thus allowing peroxide access to the active site. These structural studies further suggested that a tryptophan residue found in the hydrophobic cavity at the interface of the low and high potential domains (Trp-94, *Pseudomonas* numbering) is a possible electron transfer conduit between the two heme groups because its indole ring interacts with the propionate groups of the two hemes. To date, the most thoroughly studied BCCPs are those from *P.
Structure and Mutagenesis of R. capsulatus CCP

*Pseudomonas aeruginosa* and *Paracoccus pantotrophus* (5, 8, 11–21). Spectroscopic studies of these enzymes suggest the existence of a complex reaction mechanism that involves changes in the redox and spin states of the heme groups. In both cases, the completely oxidized CCP is inactive, whereas the mixed valence state reacts rapidly with hydrogen peroxide.

In the completely oxidized enzyme the high potential electron-transfer C-terminal heme group is in high spin/low spin equilibrium and is ligated by a histidine and a methionine (14). The second heme is a low potential group bound to the N-terminal domain and is coordinated by two histidines in the oxidized form (8). Further studies of these two enzymes and of those of *P. nautica* and *R. capsulatus* revealed a dependence on Ca\(^{2+}\) ions, although the requirement of this ion for the function of *P. aeruginosa* CCP has not been fully investigated. However, the crystal structure of *P. aeruginosa* CCP does contain bound calcium even without the addition of the ion to any of the media used during purification and crystallization of the enzyme, suggesting that this BCCP also has an inherent affinity for calcium (8). In the current model of the reaction mechanism, reduction of the high potential heme results in a switch of the low potential heme to a high spin state, and calcium is essential for this switch. In the mixed-valence form, the sixth heme ligand of the N-terminal heme is released from the iron, and hydrogen peroxide can enter the active site to be reduced at the peroxidatic center.

In almost all peroxidases a catalytic distal histidine is essential for promoting the heterolytic cleavage of the peroxide O-O bond. In the structure from the OUT-form of the *P. nautica* CCP and in that of the *N. europaea* CCP, His-74 (the sixth heme ligand of the NT heme in the complete oxidized form) is at a position where functioning as an acid-base catalyst is impossible. In chloroperoxidases a glutamic acid acts as an acid-base catalyst and helps to promote heterolytic cleavage of the peroxide O-O bond to form compound I (22–24). In the *N. europaea* CCP structure, a conserved glutamic acid is present near the active site, which forms a ligand pocket for the peroxide. Through its charge, arginine makes the distal side more polar and helps to “pull” the O-O bond of the bound peroxide by stabilizing the separating charge.

In the present paper we have determined the crystal structure of the *R. capsulatus* enzyme to compare it with the known structures of BCCPs and to complement the structure with site-directed mutagenesis studies. So far, proposals for the general mechanism of bacterial CCPs were only based on insights derived from spectroscopic and structural studies, whereas the field lacked site-directed mutagenesis data. In this respect our combined approach helps to evaluate and complement previous mechanistic proposals and to provide new insights.

### EXPERIMENTAL PROCEDURES

**Crystallization and Structure Determination**—Recombinant *R. capsulatus* CCP was prepared and crystallized as previously described (7, 25). Briefly, the periplasmic fraction was loaded onto a Q-Sepharose column and eluted with a step gradient of 0–500 mM NaCl. The fractions between 0.2 and 0.3 M NaCl were pooled and concentrated using ammonium sulfate precipitation. The BCCP-containing fractions were separated on an octyl-Sepharose hydrophobic interaction column. As a polishing step, the BCCP was subjected to anion-exchange chromatography. The enzyme fractions were pure according to SDS-PAGE and mass-spectrometric analysis (7).

Crystals suitable for x-ray diffraction analysis were obtained from 25% (w/v) polyethylene glycol monomethyl ether 550, 100 mM MES, pH 6.5, 0.01 M ZnSO\(_4\); they appeared after 2–3 weeks at 294 K.

Cryostal of RcCCP belong to space group P2\(_2\)2\(_1\)2\(_1\) with unit-cell parameters \(a = 64.6\) Å, \(b = 132.5\) Å, and \(c = 163.9\) Å and contained four molecules of RcCCP in the asymmetric unit of the crystal. The crystals diffracted poorly using our in-house x-ray source (FR591 rotating-anode generator, Bruker-Nonius) but yielded a complete data set to 2.7 Å at beamline X11 (EMBL-DESY, Hamburg) (Table 1). The structure was determined by molecular replacement methods with the program CNS (26) using the *P. aeruginosa* CCP structure (8) as a search model, with non-conserved amino acid residues mutated to alanines. PaCCP emerged as the most suitable search model following structure-based sequence alignments of CCPs of known structure against the sequence of RcCCP. Initially, plausible rotation and translation functions for one RcCCP protomer were identified and subsequently used to search for the remaining three molecules of RcCCP in the crystal asymmetric unit. As expected, the four molecules were arranged as two dimers. Placement of the model was optimized by rigid-body refinement. The correctness of the structure solution was assessed from the quality of the electron density for missing side chains and other unique structural features. Several rounds of model building were subsequently alternated with further crystallographic refinement employing simulated-anneling, conjugate-gradient minimization, and individual B-factor refinement using a maximum-likelihood target function as implemented in CNS (26) (Table 1). Non-crystallographic symmetry restraints were applied to main-chain atoms and were optimized throughout refinement to account for the higher structural mobility in loop regions and elsewhere in the structure. The structure contains eight well ordered Zn\(^{2+}\) ions at crystal contact regions, consistent with the absolute requirement for ZnSO\(_4\) in obtaining diffraction-quality crystals. The stereochemical correctness of the final model was assessed with the program MOLPROBITY (27).

### TABLE 1

| Data collection | P2\(_2\)2\(_1\)2\(_1\) | DESY/X11, 1 Å |
|----------------|------------------|----------------|
| Source, wavelength | 414706/39539 | 95.2 (95.7) |
| Completeness (%) | 12.5 (39.4) | 9.2 (3.1) |
| Resolution (Å) | 0.249 (0.372), 0.278 (0.408) |
| Number of reflections (working set/test set) | 51/46/45/47 |
| Molecules per asymmetric unit | 48/45/36, 43 |
| Protein atoms | 41, 40, 43, 41 |
| Heme-group atoms | 40/46/54 |
| Water molecules | 26 |
| Zn\(^{2+}\) (Å) | 8 |
| B-values (Å\(^2\)) | 1.4 |
| Protein (chains A/B/C/D) | 1.5/2.2 |
| Molecules per asymmetric unit | 4 |
| Number of reflections (total/unique) | 39539 |
| Source, wavelength | DESY/X11, 1 Å |
| Reflections (total/unique) | 414706/39539 |
| Resolution (Å) | 30–2.7 |
| Rsym | 0.009 |
| Angles (°) | 1.4 |
| B-values (Å\(^2\)) (main chain/side chain) | 1.5/2.2 |

*\(I_0\) is the observed intensity, and \(I\) is the average intensity for symmetry-related reflections.*

Crystals of RcCCP belong to space group P2\(_2\)2\(_1\)2\(_1\) with unit-cell parameters \(a = 64.6\) Å, \(b = 132.5\) Å, and \(c = 163.9\) Å and contained four molecules of RcCCP in the asymmetric unit of the crystal. The crystals diffracted poorly using our in-house x-ray source (FR591 rotating-anode generator, Bruker-Nonius) but yielded a complete data set to 2.7 Å at beamline X11 (EMBL-DESY, Hamburg) (Table 1). The structure was determined by molecular replacement methods with the program CNS (26) using the *P. aeruginosa* CCP structure (8) as a search model, with non-conserved amino acid residues mutated to alanines. PaCCP emerged as the most suitable search model following structure-based sequence alignments of CCPs of known structure against the sequence of RcCCP. Initially, plausible rotation and translation functions for one RcCCP protomer were identified and subsequently used to search for the remaining three molecules of RcCCP in the crystal asymmetric unit. As expected, the four molecules were arranged as two dimers. Placement of the model was optimized by rigid-body refinement. The correctness of the structure solution was assessed from the quality of the electron density for missing side chains and other unique structural features. Several rounds of model building were subsequently alternated with further crystallographic refinement employing simulated-anneling, conjugate-gradient minimization, and individual B-factor refinement using a maximum-likelihood target function as implemented in CNS (26) (Table 1). Non-crystallographic symmetry restraints were applied to main-chain atoms and were optimized throughout refinement to account for the higher structural mobility in loop regions and elsewhere in the structure. The structure contains eight well ordered Zn\(^{2+}\) ions at crystal contact regions, consistent with the absolute requirement for ZnSO\(_4\) in obtaining diffraction-quality crystals. The stereochemical correctness of the final model was assessed with the program MOLPROBITY (27).
Site-directed Mutagenesis for Substitutions H74M, W97A, W97F, Q107A, E117A, E117K, E117H, M118H, M118L, and M278H in R. capsulatus CCP—Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and were verified by DNA sequencing. PCR was carried out in a 50-μl mixture using 50-ng template plasmid DNA (pMC-ccp) (7), 125 ng of each primer carrying the mutation, 10 nmol of dNTPs, and 2.5 units of Pfu Turbo DNA polymerase (Stratagene) in Pfu polymerase reaction buffer. The thermal cycler was programmed as follows: initial denaturation at 95 °C for 30 s, 18 cycles at 95 °C for 30 s, 52 °C for 1 min, and 68 °C for 10 min. One microliter (20 units) of DpnI (New England Biolabs) was added to the sample (50 μl) and incubated at 37 °C for 1 h. Two microliters of the DpnI-treated PCR reaction was used to transform competent Escherichia coli cells (XL1-Blue). The mutant proteins were overexpressed in E. coli and purified as described previously (7). The folding was verified by gel filtration chromatography on a HiLoad 16/60 Superdex 75 prep (Biosciences) column using 100 mM Tris-HCl, pH 7.4, 100 mM NaCl as eluant. All mutant enzymes eluted at a volume corresponding to a dimer.

Peroxidase Assay—R. capsulatus bacterial cytochrome c peroxidase can use mitochondrial cytochrome c and R. capsulatus cytochrome c₅₆ as electron donors (7). The activity of the enzyme was monitored through the decrease in absorbance of the α-band of the ferrocytochrome c at 550 nm. The assay mix was composed of 10 mM Hapes, pH 7.5, 1 mM CaCl₂, 6 μM ferrocytochrome c, and 18 μM H₂O₂, and was stirred continuously (3). The reaction was started through the addition of the enzyme to a final concentration of 1.3 mM. The reaction is first order toward horse ferrocytochrome c under conditions where the reaction is rapid. Under such conditions, the initial velocity (v) can be calculated by determining the pseudo-first-order rate constant (k) and by use of the relationship v = k[C₅₆], where C₅₆ is concentration of the ferrocytochrome.

Potentiometric Redox Titrations—The titrations were performed as described previously (7).

Cyanide Titrations—A volume of 500 μl of 4 μM protein in 10 mM Hapes, pH 7.5, was titrated by the addition of different amounts of a neutralized sodium cyanide solution. The wild-type enzyme and M118L mutant were titrated with 0, 1, 3, 5, 25, 65, 105, 205, and 305 μM sodium cyanide, and the M118H mutant was titrated with 0, 1, 3, 5, 105, 205, 305, 405, and 505 μM sodium cyanide. The titration was carried out on the calcium-activated mixed-valence state of the wild-type and mutant peroxidase.

Structural Alignments and Figure Preparation—Structures of CCP homologs were superimposed using the program SEQUOIA (28). Figs. 1 and 7 were prepared and rendered with PyMOL Version 0.98 (www.pymol.org).

RESULTS AND DISCUSSION

Overview of the Structure—The crystal structure of the fully oxidized RecCP at 2.7 Å of resolution reveals a 2-domain α/β structure reminiscent of other structurally characterized CCPs (8–10) (Fig. 1A). The tertiary structure is organized into two domains that stack on top of each other and create an interaction interface of about 1200 Å (2). Each domain features a classical c-type cytochrome fold (29) and harbors a covalently bound heme center. The low potential domain (LP) is almost entirely helical and is defined by residues 20–167 and 306–326. It contains a hydrophobic pocket that binds the low potential heme center via the heme binding motif Cys-54–X₇–Cys-57–His-58…His-74, with His-58 and His-74 being the axial ligands. On the other hand, the high potential domain (HP) contains a more balanced mixture of secondary structure and is formed by residues 4–19 and 168–305. The HP domain has His-204 and Met-278 as the axial ligands to the heme and employs the heme binding motif Cys-200–X₇–Y=Cys-203–His-204…Met-278 to bind to the heme center. The two iron ions are about 20 Å from each other.

The interface between the two domains takes the form of a hydrophobic cavity and accommodates a calcium ion that is equidistant (12 Å) from the iron centers of the LP and HP heme groups. Based on temperature factor (β-factor) comparisons, this Ca²⁺ is present at full occupancy in all four molecules of RecCP in the asymmetric unit of the crystal and is coordinated by five ligands: Asn-82 O6i, Thr-259 C=O, Pro-261 C=O, and two water molecules (Fig. 1B). This geometry is analogous to that observed in the PaCCP and PnCCP-OUT structures (8, 10) but is lacking two additional water molecules between the calcium and the propionate of the high potential heme group that help complete the distorted pentagonal bipyramidal geometry seen in the other two structures. As in PaCCP and PnCCP, the charge of the calcium ion in RecCP is not countered by negatively charged groups, suggesting that it can act as a modulator of electron transfer between the two heme domains.

Structural Comparisons with Other CCP Structures—Pairwise structural overlays of RecCP against PaCCP (8) and the IN-form of PnCCP (10) reveal that the three enzymes are very similar overall, as expected from their high sequence identity (RecCP versus PaCCP: 1.34 Å r.m.s.d. for 293 Ca atoms; RecCP versus PnCCP-in: 1.58 Å r.m.s.d. for 297 Ca atoms) (Fig. 1C). Rather unexpectedly, however, the three structures exhibit significant structural differences in the LP domain and, more specifically, in the structural segment (defined by residues 108–118 in RecCP) just adjacent to the flexible loop that contributes His-74 as a ligand to the low potential heme (Fig. 1D). During catalysis this loop loses its ligation to the low potential heme group via His-74 and swings outward to make room for the incoming peroxide (9–10). In PaCCP, the equivalent segment to residues 108–118 is well ordered and forms a helix (Fig. 1D). In PnCCP-IN this segment is significantly more extended with much less secondary structure and higher B-factors (Fig. 1D). In RecCP, however, this segment is completely disordered. Crystal packing analysis revealed that residues 108–118 in RecCP in all four molecules in the asymmetric unit of the crystal are not involved in crystal contacts. Similar analyses for the other two enzymes indicated the same feature. RecCP is the only enzyme of the three considered here that has been crystallized with more than one molecule in the asymmetric unit of the crystal and, therefore, offers an opportunity to visualize the intrinsic conformational diversity of the molecule. Pairwise superpositions of these four crystallographically observed RecCP molecules reveal overall r.m.s.d. values between 0.27 and 0.4 Å for all Ca atoms, indicating a strong conservation of conformational space. Interestingly, the most pronounced local structural differences concern residues 104–107 (1.8 Å r.m.s.d., i.e. the four residues preceding the disordered loop. Taken together, our structural analyses indicate that the degree of flexibility in the segment described by residues 108–118 obeys the trend PaCCP < PnCCP < RecCP, suggesting that this may be of functional relevance.

Expression and Purification of the Mutant Enzymes—The expression and purification protocol of the wild-type enzyme has been previously described (7). For the purposes of our mutagenesis studies we have used the expression construct for the wild-type enzyme as the basis to create and express site-directed mutants. The mutant enzymes were overproduced and purified in the same way as the wild-type enzyme. Correct folding of the mutant enzymes was checked by gel filtration experiments and via the UV-visible spectrum of the protein. Optical spectra of the heme proteins are very sensitive to structural changes around the heme.
FIGURE 1. Structure of RcCCP and comparisons with other CCPs. A, stereo view of the overall structure and location of the mutated residues as is discussed under "Results and Discussion." B, details of the calcium binding site. wat, water. C, stereo view of a structural superposition of RcCCP (green) with PaCCP (red, PDB code 1EB7) and PnCCP-IN (blue, PDB code 1RZ6). D, close-up view of the flexible structural segments in the region of the low potential heme.
and can, therefore, be used to probe structural changes due to protein unfolding. The gel filtration experiments revealed that all the produced recombinant mutants are dimers.

Mutagenesis and Mechanistic Considerations: Structural Flexibility Near the Low Potential Heme Is Important for Activity—Interestingly, the highly mobile segment of RcCCP described above accommodates the catalytic important Glu-117 near the peroxide binding site at the LP domain. Glu-117 has been proposed to act as the acid-base catalytic group required for cleavage of the peroxide bond (9). Site-directed mutagenesis of this residue to a leucine results in an inactive enzyme with a low spin, implicated in interactions with diatomic ligands (9). Substitution of this residue with leucine also resulted in an inactive enzyme with a low spin, preventing access of hydrogen peroxide. Located at the N-terminal end of the 108–118 flexible region is Gln-107, which has been implicated in interactions with diatomic ligands (9). Substitution of this residue with leucine also resulted in an inactive enzyme with a low spin, low potential heme in the mixed valence and in the completely reduced state (Table 2A). Furthermore, PaCCP and RcCCP, the two enzymes with, respectively, the most and least structured segment for residues 108–118, were both crystallized from polyethylene glycol solutions at 25% (w/v) at physiological pH.

To investigate in more detail the acid-base profile of Glu-117, we mutated it to histidine and lysine, expecting to see a pH-dependent change in activity. The enzymatic activity of RcCCP shows a bell-shaped curve, with an optimum at pH 7 and pK values of 6.1 and 7.9 (30). The curve may be understood in terms of the structure of the protein in the vicinity of the two hemes. The ionizations leading to the pK values may be due to histidine 74 and 264, to glutamate 117, or to any of the four heme propionates. The Glu-117 mutants, however, were inactive over the complete pH range, and we could detect neither a high spin signal in the mixed valence/calcium-activated state nor in the fully reduced form. This indicates that the peroxidatic iron remains bound to a strong field ligand, preventing access of hydrogen peroxide. Located at the N-terminal end of the 108–118 flexible region is Gln-107, which has been implicated in interactions with diatomic ligands (9). Substitution of this residue with leucine also resulted in an inactive enzyme with a low spin, low potential heme in the mixed valence and in the completely reduced state (Table 2A). This again shows that the peroxidatic heme remains coordinated by a strong field ligand and implies that the axial ligand His-74 does not swing away to create space for binding the hydrogen peroxide.

Coordination of the Low and High Potential Heme Groups—In the UV-visible spectrum of the fully oxidized but inactive state of the wild-type enzyme, both 380 and 620 nm bands are present, which indicates that one of the two heme groups is in a low spin/high spin equilibrium (Fig. 3). Visible and near-IR magnetic circular dichroism at 4.2 and 290 K and EPR spectroscopy at low temperature of the oxidized PaCCP have revealed the presence of a high potential heme with the unusual properties of being ligated by methionine and histidine in that the methionine ligand appears to be more loosely bound than is usual for a cyto-

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**TABLE 2**

| Protein          | Redox potential | Oxidized state | Ascorbate reduced state | Calcium-activated and ascorbate reduced state | Dithionite reduced state | % Activity |
|------------------|-----------------|----------------|-------------------------|-----------------------------------------------|--------------------------|------------|
| Wild type        | −270 mV         | HS/Ls          | HS                      | HS                                            | LS                       | 100        |
| Sixth heme ligands |                 |                |                         |                                               |                           |            |
| H74M             | +230 mV         | HS/Ls eq       | HS heme reduced         | −LS                                           | LS                       | 0          |
| M278H            | −180 mV, −215 mV| 610 nm band    | 610 nm band             | 610 nm band                                  | 610 nm band              | 0          |
| Amino acids on the flexible loop |        |                |                         |                                               |                           |            |
| Q107L            | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| M118H            | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| M118L            | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| E117L            | HS/Ls eq        | HS heme reduced | HS                      | HP heme reduced                               | LS                       | 0          |
| E117H            | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| E117K            | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| Amino acids lying between the two heme groups |        |                |                         |                                               |                           |            |
| W97A             | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | 0          |
| W97F             | HS/Ls eq        | HS heme reduced | LS                      | HP heme reduced                               | LS                       | <1         |

**TABLE 3**

| Enzyme          | Physiological electron donor (concentration) | Turnover number |
|-----------------|---------------------------------------------|-----------------|
| R. capsulatus (7)| µM                                         | µs              |
| P. aeruginosa (13)| 6                                          | 1060            |
| P. nautical (6)   | 12                                         | 90              |
|                 | 12                                         | 181             |
The results did not allow us to differentiate between two possible scenarios, 1) an equilibrium between a six- and a five-coordinate species with and without an axial methionine and 2) an equilibrium between two six-coordinate heme groups, one with methionine and the other with a weak-field ligand supplied by the protein or by solvent (4, 14). To shed light on this heme-group coordination, we mutated His-74, the sixth ligand of the N-terminal heme group, to methionine and in a separate experiment, Met-278, the sixth heme ligand of the C-terminal heme, to histidine.

We had expected that the H74M would result in an enzyme with two methionine-histidine-ligated high potential heme groups. The redox potential of the heme groups were determined to be \(-180\) and \(-230\) mV, respectively, which indicates that the introduced methionine does not ligate the N-terminal heme. The fully oxidized mutant enzyme still showed a 380- and 620-nm band, suggesting that the C-terminal heme group is responsible for this high spin signal. Another peculiarity of this mutant is the presence of a broad band at 610 nm in all oxidation states. Nitrate reductase possesses a similar 610-nm band that originates from a high spin heme (31). In cytochrome \(aa_3\) oxidase from Paracoccus denitrificans, a 610-nm band is formed when an oxy-ferryl center is formed after reaction of \(H_2O_2\) with the oxidized enzyme. The mixed valence oxidase also forms a 610-nm band after reacting with oxygen (32). It is possible, therefore, that the 610-nm band in the H74M mutant arises from the reaction of the enzyme with oxygen, resulting in an inactive species. On the other hand, this band can be caused by a high spin heme, which means that the N-terminal heme group would be in a high spin conformation in all oxidation states, a feature that can be induced by structural perturbations in the mutant enzyme. Moreover, the mutant enzyme is inactive, which is in accordance with the absence of the 380 nm band in the ascorbate-reduced and calcium-activated form.

The M278H mutant behaved as expected and contains two low potential hemes (\(-215\) mV and \(-105\) mV), indicating that both heme groups are bis-histidine-ligated. Moreover, this mutant does not exhibit high spin bands at 380 and 630 nm in the oxidized state, which suggests that it is the C-terminal heme that contributes to the high spin/low spin equilibrium in the native enzyme (Fig. 3). We can, therefore, conclude...
that the C-terminal heme group exists in an equilibrium between a hexa- and a penta-coordinated state.

**Insights into the Mechanism of H₂O₂ Reduction**—The mechanism of H₂O₂ reduction in the BCCPs is not yet clearly understood. Spectroscopic and electrochemical studies from several of these bacterial peroxidases have indicated that, upon reduction of the high potential heme and in the presence of calcium, the enzyme becomes accessible to hydrogen peroxide. However, it has so far been unclear which heme group acts as the peroxidatic center and which one acts as the electron transferring heme (4, 33, 34). Two plausible mechanisms have been proposed to date (35, 36) and are represented in Fig. 4. In the first model, only a spin state change is assumed to occur. In the starting state, the electron transferring heme is C-terminal, whereas the peroxidatic center is the low potential, N-terminal heme. The latter would switch from low spin to high spin, which reflects its accessibility to solvent after removal of the sixth ligand. In the second model, both a spin switch and a ligand switch would occur. In the LP-heme, His-74 would be replaced by Met-118, whereas the HP-heme would lose its sixth ligand (Met-278). Structural data from several bacterial cytochrome c peroxidases and, in particular, recent studies on the _P. nautica_ enzyme favor the first model (10). In that study the structure of the active form of PnCCP, termed PnCCP-OUT, reveals that the N-terminal heme group is penta-coordinated.

To gain additional insights into the mechanism of peroxide reduction we subjected the key residue, Met-118, to site-directed mutagenesis. When Met-118 was mutated to a histidine, the enzyme was inactive. The C-terminal heme group in the fully oxidized state is still in a high spin/low spin equilibrium, just like the wild-type enzyme. However, neither the calcium-activated, mixed valence state nor the fully reduced form showed a high spin signal, a 380-nm band, and unresolved α and β peaks (Fig. 5). Together, these characteristics support the inactivity of the mutant. In PnCCP-OUT, Met-118 packs rather non-specifically via van der Waals interactions against the N-terminal heme group in a broad hydrophobic pocket. It, thus, appears that histidine in the RccCP mutant is too polar to be able to compensate for the function of Met-
118. Alternatively, histidine in the M118H could act as a sixth ligand to the N-terminal heme group but only after the displacement of His-74 in the OUT position (10). To distinguish between these two possibilities, Met-118 was mutated to a leucine, because this residue type is more similar in size and hydrophobicity to methionine and cannot function as a sixth heme ligand. The M118L mutant behaved spectroscopically like the M118H mutant, except that some calcium effect, seen at 380 nm, was detectable and the peaks of the reduced low potential heme were not well resolved (Fig. 5). Both signals were smaller compared with the wild-type signals, which suggests that at least some of the enzyme species can adopt a high spin state, explaining the residual, but 14 times smaller, activity compared with the wild-type enzyme. Cyanide titrations were carried out to assess the accessibility of one of the hemes (Fig. 6). At low concentrations of cyanide (≤1 μM), the high spin heme group of the M118H mutant is less accessible than the heme in the M118L mutant, and this in turn is less accessible than that of the wild-type enzyme. At high concentrations of cyanide the wild-type enzyme and mutants react with the anion. We, therefore, conclude that the heme
group of the wild-type enzyme is most accessible and that the heme of the Leu mutant is more accessible than that of the His mutant. This reflects a more open structure in the vicinity of the N-terminal low potential heme group of the wild-type enzyme followed by the Leu mutant and the more closed conformation of the His mutant even though this mutant reacts similarly to the Leu mutant at high concentrations of cyanide. These observations lend further support to the much higher turnover number of the wild-type enzyme. The equilib-

Electron Transfer Pathway—An important step in the reduction of hydrogen peroxide is that the electrons need to be transferred from their point of entry at the C-terminal high potential heme to the N-terminal low potential heme and eventually to $\text{H}_2\text{O}_2$. As our structural analysis has shown, the two heme groups are separated by an iron-iron distance of $\sim 10$ Å and are positioned perpendicular to each other such that a propionate from the high potential heme approaches a propionate from the low potential heme at a distance of $\sim 10$ Å, with the indole ring of Trp-97 located in between (Fig. 7). This residue has been proposed to act as a conduit for electron transfer between the two hemes in the $P$. aeruginosa, $N$. europeae, and the $P$. nautica enzymes (8–10). Trp-97 lies in the plane of the high potential heme but is perpendicular to the low potential heme. It is known that electron transfer between redox centers in proteins can occur over distances up to 14 Å through the mechanism of electron tunneling (38). Amino acids that can exist in radical forms, such as tyrosine or tryptophan, can form the necessary chain elements for such electron tunneling phenomena. When we mutated Trp-97 to an alanine, RccCP became completely inactive, whereas a W97F mutation yields an enzyme with residual activity about 100 times less that of the wild-type enzyme. Spectroscopically, the two mutants are quite different. W97A is completely low spin in the fully reduced state, whereas W97F is partially high spin in this state. Furthermore, the phe-

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