Oxygen-dependent Coproporphyrinogen III Oxidase (HemF) from Escherichia coli Is Stimulated by Manganese*

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During heme biosynthesis in Escherichia coli two structurally unrelated enzymes, one oxygen-dependent (HemF) and one oxygen-independent (HemN), are able to catalyze the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX. Oxygen-dependent coproporphyrinogen III oxidase was produced by overexpression of the E. coli hemF in E. coli and purified to apparent homogeneity. The dimeric enzyme showed a $K_m$ value of 2.6 $\mu$M for coproporphyrinogen III with a $k_{cat}$ value of 0.17 min$^{-1}$ at its optimal pH of 6. HemF does not utilize protoporphyrinogen IX or coproporphyrin III as substrates and is inhibited by protoporphyrin IX. Molecular oxygen is essential for the enzymatic reaction. Single turnover experiments with oxygen-loaded HemF under anaerobic conditions demonstrated electron acceptor function for oxygen during the oxidative decarboxylation reaction with the concomitant formation of H$_2$O$_2$. Metal chelator treatment inactivated E. coli HemF. Only the addition of manganese fully restored coproporphyrinogen III oxidative activity. Evidence for the involvement of four highly conserved histidine residues (His-96, His-106, His-145, and His-175) in manganese coordination was obtained. One catalytically important tryptophan residue was localized in position 274. None of the tested highly conserved cysteine (Cys-167), tyrosine (Tyr-135, Tyr-160, Tyr-170, Tyr-213, Tyr-240, and Tyr-276), and tryptophan residues (Trp-36, Trp-123, Trp-166, and Trp-298) were found important for HemF activity. Moreover, mutation of a potential nucleotide binding motif (GGGXTP) did not affect HemF activity. Two alternative routes for HemF-mediated catalysis, one metal-dependent, the other metal-independent, are proposed.

Tetrapyrroles, like the porphyrins heme and chlorophyll, are essential compounds of the metabolism of almost all organisms on earth. They are integral parts of the electron transport chains utilized during photosynthesis and oxidative phosphorylation. Moreover, various enzymes utilize tetrapyrrole cofactors.

During porphyrin biosynthesis coproporphyrinogen III oxidase (CPO, EC 1.3.3.3) catalyzes the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrin IX (protogen) (Fig. 1). In Escherichia coli two structurally unrelated CPOs, one oxygen-dependent (HemF) and one oxygen-independent (HemN), catalyze this reaction (1, 2). The enzymatic mechanisms for both reactions are subjects of controversy. Initial reports on the path of coprogen decarboxylation were obtained using deuterium- and tritium-labeled propionate side chains of the substrate (3). The enzymatic decarboxylation of ring A always occurs prior to that of ring B, which was shown through the isolation of the intermediate harderoxy coproporphyrinogen (4). These experiments suggested an anti-parallel elimination as part of the mechanism for the formation of the two vinyl groups. Three possible mechanisms for the oxygen-dependent vinyl group formation were proposed. One mechanism involves an oxygen-dependent hydroxylation step. The other two mechanisms are variants of the same basic idea, in that formation of the vinyl groups results in the simultaneous hydride abstraction and decarboxylation (5). A radical based mechanism was recently elucidated for the oxygen-independent CPO HemN from E. coli (6). This enzyme belongs to the “radical S-adenosylmethionine family” of enzymes (7).

For the interaction of enzymes with molecular oxygen nature often employs metal ions. However, there are conflicting reports on the metal ion contents of various oxygen-dependent CPOs (HemF). Purified recombinant mouse HemF was described to contain copper, native yeast HemF iron and recombinant human HemF were found metal-free (8–11). Surprisingly, no significant influence of the various found metal ions on the activity of the different analyzed CPOs was detected. HemF proteins from plant, animal, and bacterial sources possess an amazingly high conserved amino acid sequence (Fig. 2).

First results from site-directed mutagenesis approaches using mouse HemF indicated an involvement of one highly conserved histidine residue in catalysis (8). Moreover, the importance of tyrosine residues was proposed as potential location for a hypothetical tyrosyl radical (12).

Recently, common structural properties were proposed for the oxygen-dependent CPO and urate oxidase (13). Both enzymes utilize oxygen. Based on the assumption that HemF performs a cofactor- and metal ion-independent catalysis a hypothetical enzyme structure was modeled that places HemF into the new family of T-fold proteins.

Here we describe the biochemical and biophysical characterization of purified recombinant E. coli CPO. The activity of the enzyme is stimulated by manganese. Molecular oxygen serves as an electron acceptor with results in the generation of H$_2$O$_2$. Catalytically important amino acid residues were identified using site-directed mutagenesis.

IX, protogen, protoporphyrinogen IX; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide HCl; MES, 4-morpholinethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Construction of the E. coli hemF Expression Vector pCalhemF—A DNA fragment encoding all amino acid residues of E. coli HemF was generated by polymerase chain reaction from genomic DNA using primer 1 (5'-H11032-GAAAAAGGGATCCGTTATGAAACC-3' /H11032) and primer 2 (5'-H11032-AGCGGATCCTCGAGTGGGGG-3' /H11032). The resulting DNA fragment was digested with BamHI and XhoI (recognition sequences underlined in the primer sequences) and ligated in the appropriately digested vector pCal-n (Stratagene, Heidelberg, Germany) to generate pCalhemF, which is under control of a T7 RNA polymerase promotor. The integrity of the plasmid was confirmed by DNA sequencing. The resulting fusion protein with a calculated molecular mass of 38,325 Da consists of the N-terminal calmodulin-binding peptide (CBP) of 4,000 Da fused via a thrombin recognition sequence to E. coli HemF of 34,325 Da. After proteolytic cleavage of the fusion protein with thrombin, three additional amino acids (VPR) instead of the original methionine are localized at the N terminus of HemF.

Site-directed Mutagenesis of E. coli hemF—Mutant pCalhemF plasmids encoding E. coli hemF with amino acid exchanges H96L, H106L, H145L, H175L, C167S, Y135F, Y160F, Y170F, Y213F, Y240F, W36L, W123L, W166L, W274L, W298L, W124R, G127V, T132A, and P133A were constructed using the QuikChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The primer used to generate the mutations were: H96L forward, 5'-H11032-GGCGTTTCACTGGTAGTGCTTC-CGCATAACCCG-3' /H11032; H106L forward, 5'-H11032-CCCACCAGCCTCGCGAATGGCGG-3' /H11032; H145L forward, 5'-H11032-GCTATTCACTGGCTTCGCACCGCG-3' /H11032; H175L forward, 5'-H11032-CTTCTACCTCAAACTTCGCAACGAACAGCG-3' /H11032; C167S forward, 5'-H11032-CCCCGTTACAAAAAGTGGTCCGACGAATACTTCTAC-3' /H11032; Y135F forward, 5'-H11032-CGACTTAACCCCATTCTTTGGTTTTAAGAAGAT-3' /H11032; Y160F forward, 5'-H11032-CCATTTGGCGAAGACGTTTTTCCCGTTACAAAAAG-3' /H11032; Y170F forward, 5'-H11032-GTTGTGCGACGAATTCCTTTTTTACCGCTGGTAATCT-3' /H11032; Y213F forward, 5'-H11032-GCTACACCGACGCTTTTTTACCAATTGTCGAGCG-3' /H11032; Y240F forward, 5'-H11032-CGCGGTCGGTTTTGTCGAGTTCAATCTGGTCTGG-3' /H11032; Y276F forward, 5'-H11032-CTGGTAACGCTGGGAATTTGATTATCAGCCAAAAGATG-3' /H11032; W36L forward, 5'-H11032-GTCGAAGATAGTTTGCAGCGCGAAGCTG-3' /H11032; W123L forward, 5'-H11032-CGATCCCGTCTTGTGGTTTGGCGGTG-3' /H11032; W124R forward, 5'-H11032-GATCCCGTCTGGAGGTTTGGCGGTGGC-3' /H11032; W166L forward, 5'-H11032-CCCCGTACAAAAAGTTGTGCGACGAATACTTC-3' /H11032; W274L forward, 5'-H11032-GACGCTGGGAATTTGATTATCAGC-3' /H11032; W298L forward, 5'-H11032-GGACGCTGGGAATTTGATTATCAGC-3' /H11032; G127V forward, 5'-H11032-CTGACGCTGGGAATTTGATTATCAGC-3' /H11032; T132A forward, 5'-H11032-GCTTCGA-CTTAACCGCATTCTATGGTTTTG-3' /H11032, P133A forward, 5'-H11032-GCTTCGA-CTTAACCGCATTCTATGGTTTTG-3' /H11032. All mutated hemF genes were subjected to complete DNA sequence determination.

Overexpression of E. coli hemF and Purification of HemF—E. coli BL21(DE3) cells carrying the wild type or mutated pCalhemF were grown in LB medium at 37 °C with shaking at 200 rpm (14). When the cultures reached an A578 of 0.7 protein production was induced by addition of 100 μM isopropyl-1-thio-β-D-galactopyranoside. The cells

FIG. 1. Scheme of the reaction catalyzed by HemF. The educt coprogen is converted in a two-step reaction into protogen. The two propionate groups at rings A and B are oxidatively decarboxylated to vinyl groups with the release of two molecules CO2.

FIG. 2. Amino acid sequence alignments of conserved regions of vertebrate, plant, and bacterial HemFs. Amino acid exchanges introduced into E. coli HemF via site-directed mutagenesis are indicated.
were further cultivated at 30 °C overnight, harvested, washed three times using buffer A (50 mM Tris/HCl, pH 7.2, 200 mM NaCl, 2.5 mM MnCl₂, 0.1% Triton X-100), and resuspended in a minimal volume of buffer A. Cells were broken under strict anaerobic conditions as outlined above with the exception of the cation were identical to what is outlined above with the exception of the.

were incubated with calmodulin affinity material (Amersham Biosciences) at 4 °C with rigorous shaking overnight for efficient CBP-HemF binding. After washing three times with buffer A the bound CBP-HemF fusion protein was eluted in 10 ml of buffer B (50 mM Tris/HCl, pH 8.0, 1 m NaCl, 0.1% Triton X-100 including 10 mM calmodulin inhibitor W7, obtained from Sigma). After concentration and desalting of the CBP-HemF solution the CBP tag was cleaved off using 50–70 units of thrombin protease per mg of fusion protein in buffer C (50 mM Tris/HCl, pH 8.3, 200 mM NaCl). The CBP of 3,646 Da was removed from E. coli HemF via dialysis using dialysis tubing with a molecular weight cut-off of 20,000. The homogeneity and integrity of the purified HemF was determined by SDS-polyacrylamide gel electrophoresis, N-terminal protein sequencing, and mass spectrometry.

Determination of Protein Concentration—The BCA (bicinchoninic acid) protein assay kit (Pierce) was used according to the manufacturer’s instructions using bovine serum albumin as a standard.

Determination of the Native Molecular Mass of HemF—The relative native molecular mass of HemF was determined using gel permeation chromatography and glycerol gradient centrifugation as previously described (15). Isoelectric focusing electrophoresis was performed as outlined before (15).

UV-Visible Light Absorption Spectroscopy—UV-visible absorption spectra (190 to 730 nm) were recorded using a PerkinElmer Lambda 2 spectrophotometer as described before (6). N-terminal Protein Sequencing and Mass Spectrometry—The N terminus of the purified enzyme was determined by Edman degradation as described before (6). The molecular mass was determined using electrospray ionization mass spectrometry as outlined before (6).

Experimental Procedures

Coproporphyrinogen III Oxidase Assay Using Recombinant HemF—The substrate coprogen was generated as described previously (6). In a total volume of 500 µl of 334 mM HemF, 20 µM coprogen, 0.1% Triton X-100, 50 mM MES/HCl, pH 6.0, were incubated at 37 °C for 10–20 min under rigorous shaking at 1000 rpm in the dark. Reactions were stopped by the addition of 50 µl of H₂O₂ to oxidize generated protogen and residual coprogen. After 10–20 min oxidation the fluorescence of the produced proto was measured as described before (6). For kinetic HemF characterization a total reaction volume of 5 ml was employed. The fluorescence was detected at 630 nm (excitation 409 nm). Presented are the emission spectra from 590 to 680 nm with an excitation wavelength of 409 nm. A standard enzymatic assay was used as described under “Experimental Procedures.” The thin line represents the fluorescence spectrum of proto produced by HemF from coprogen and oxidized by H₂O₂ treatment. The thick line represents the fluorescence spectrum of the substrate coprogen incubated in the absence of enzyme and oxidized by H₂O₂ treatment. C, the enzymatic reaction of HemF was stopped with hydrogen peroxide immediately after mixing all components of the assay (reaction time = 0). The solution was treated as described under “Experimental Procedures” and 20 µl were injected onto the HPLC column. The fluorescence was detected at 630 nm (excitation 409 nm). Coproporphyrin elutes with a retention time of 3.01 min. If the reaction was stopped after 90 min the increased proto peak produced from HemF at a retention time of 23.7 min is clearly visible.

addition of 100 µM isopropyl-1-thio-β-D-galactopyranoside was preformed at an A₅₇₈ of 0.2. Cells were broken under strict anaerobic conditions using a French press (6). The preparation of the cell-free extract and chromatographic enzyme purification were performed in an anaerobic chamber under nitrogen atmosphere (6). Enzyme assays were performed up to 48 h under strict anaerobic conditions as outlined above.

High Performance Liquid Chromatography (HPLC) Analysis—Enzyme assays were performed as outlined above. HPLC analysis of HemF product formation was performed as described previously (6) using separation on a ODS Hypersil C18 reversed phase column (5 µm, 120 A pore width, 25-cm length, 4.6-mm inner diameter).
**RESULTS AND DISCUSSION**

Production, Purification, and Initial Biochemical Characterization of Recombinant E. coli HemF—A T7 RNA polymerase driven system was used to produce E. coli HemF as a N-terminal fusion protein with the CBP. Affinity chromatography on a calmodulin affinity matrix resulted in apparent homogeneity and integrity of recombinant purified HemF using thrombin cleavage and dialysis. The N-terminal sequence of the purified enzyme determined by Edman degradation (VPRMKPDAHQVKQFLLNZQDT) was found identical to the amino acid sequence deduced from E. coli HemF plus three additional N-terminal amino acids from the cleavage of the fusion protein.

**TABLE I**

| Preculture | Main culture | Protein purification | Enzyme assay† | Turnover‡ |
|------------|--------------|----------------------|---------------|----------|
| Aerobic    | Aerobic      | Aerobic              | Aerobic       | 21.5     |
| Anaerobic  | Anaerobic    | Anaerobic            | Anaerobic     | 0.8      |

† 334 nmol of HemF were incubated at 37 °C under standard assay conditions under aerobic or strict anaerobic conditions as outlined under “Experimental Procedures.” The reaction was started by the addition of 20 μmol of substrate. Produced product was quantified after H2O2 oxidation as shown in Fig. 4 and described under “Experimental Procedures.”

‡ Product formation was measured over a time of 90 min. The amount of product measured after this time was related to the amount of employed HemF in the enzyme assay.

Detection of H2O2 Produced during the Enzyme Reaction—Enzyme assays were performed as outlined above without adding H2O2 to stop the reaction. Detection of hydrogen peroxide produced during the enzyme reaction was performed using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes, Leiden, Netherlands).

Metal Ion Analysis—Metal analysis was performed by flame ionization atomic absorption spectroscopy using a PerkinElmer 3100 atomic absorption spectrometer. Manganese was measured at 279.5 nm, iron at 248.3 nm, and copper at 327.4 nm using a manganese, iron, or copper hollow cathode lamp. The metal standards were purchased from Merck (Germany).

**TABLE II**

| Treatment§ | Additions | HemF activity% |
|------------|-----------|----------------|
| 10 mM MnCl2 | 10 mM o-Phenanthrol | ND |
| 10 mM EGTA  | 10 mM o-Phenanthrol | ND |
| 10 mM EGTA  | 10 mM ZnCl2 | 15 |
| 10 mM EGTA  | 10 mM NiCl2 | 14 |
| 10 mM EGTA  | 10 mM FeCl2 | 6 |
| 10 mM EGTA  | 10 mM MgCl2 | 12 |
| 10 mM EGTA  | 10 mM AlCl3 | 11 |
| 10 mM EGTA  | 10 mM CuCl2 | 34 |
| 10 mM EGTA  | 10 mM MnCl2 | 60 |
| 10 mM EGTA  | 10 mM MnCl2 | 100 |
| 200 μM HgCl2 | ND            | ND |
| 200 μM PbCl2 | ND            | ND |
| 200 μM CdCl2 | ND            | ND |
| 0.5 mM SAM  | ND           | ND |
| 0.5 mM NAD  | 10 mM MnCl2  | 100 |
| 0.5 mM NADH | 10 mM MnCl2  | 75 |
| 0.02 mM copro | 10 mM MnCl2 | 90 |
| 0.02 mM proto | 10 mM MnCl2 | 110 |
| 0.02 mM DSA | 10 mM MnCl2  | 95 |

§ Purified recombinant E. coli HemF was preincubated with additions listed above for 60 min at 37 °C. Enzymatic assay were performed as described under “Experimental Procedures.” Combined EGTA and o-phenanthroline treatment fully abolished HemF activity. This treatment partially destroyed the HemF structure as detected by CD spectroscopy (data not shown). To prevent these secondary effects we tested metal dependence by single EGTA treatment.

† The fully active enzyme after MnCl2 treatment was set to 100% and all other activities were related to that.

‡ ND, not detectable.

§ Kinetic studies showed a K<sub>m</sub> = 6 μM for a mixed inhibition.

**Fig. 5. Spectroscopic characterization of recombinant E. coli HemF and various HemF mutant enzymes.** UV-visible light absorption spectra of recombinant, purified HemF proteins. Spectra were recorded for wild type HemF (A) and the mutated enzymes H175L (B), H106L (C), H96L (D), and H145L (E). The mutant H175L (B) is missing the typical absorbance spectrum for porphyrins.
determination of HemF activity. One test uses a fluorimetric and the other test a radiochemical detection. As a consequence of the different sensitivities of both test systems significant discrepancies in the measured kinetic parameters for various HemFs from various organisms were observed. The $K_m$ values for coproporphyrinogen III obtained for eukaryotic CPOs varied from $<1$ to $>10$ $\mu M$ depending on the employed assay. In this investigation a fast and sensitive fluorimetric assay was employed (17). The product of the enzymatic reaction (proto) was first chemically oxidized by hydrogen peroxide to proto, which was subsequently quantified by fluorescence spectroscopy. Using this method a $K_m$ value of 2.6 $\mu M$ and a $V_{max}$ value of 0.17 $\text{min}^{-1}$ and a $V_{max}$ value of 1.3 $\mu M$ $\text{min}^{-1}$ $\text{mg}^{-1}$ was determined for E. coli HemF and its substrate coproporphyrinogen III. Identification of the enzymatic reaction product proto was achieved using HPLC (Fig. 4). The pH optimum of the enzyme was localized at 6. This value is much lower compared with values obtained for eukaryotic CPOs that showed pH optima between 6.8 and 8.6. Recombinant HemF was unable to convert protogen to proto, which is in contrast to an earlier publication (18) that describes this oxidation for E. coli CPOs. In addition no inhibition by coprogen and proto was observed as it had been described for eukaryotic CPO (19, 20). Only the addition of 20 $\mu M$ proto significantly inhibited HemF activity, whereas all other compounds including coproporphyrin and deuteroporphyrin IX 2,4-disulfuric acid revealed no effect (see Table II). The UV-visible spectrum of purified E. coli HemF showed a Soret band at 410 nm besides the classic protein band at 283 nm. Together with 4 smaller peaks at 503, 540, 570, and 630 nm this spectrum is typical for enzyme-bound porphyrin compounds (Fig. 5, spectrum A). Control experiments including the spectral analysis of HemF incubated with various known porphyrins indicated the presence of bound protoporphyrin. The physiological relevance of this observation remains to be determined.

### Oxygen-loaded E. coli HemF Allows One Catalytic Turnover under Anaerobic Conditions

Surprisingly, the HEM13 gene of yeast and the $\text{hemF}$ gene of *Pseudomonas aeruginosa* both encoding oxygen-dependent CPOs are drastically induced under anaerobic growth conditions (22, 23). Similar findings were described for oxygen-dependent ribonucleotide reductase from E. coli (24). Later enzyme requires an aerobic activation by oxygen for the formation of a protein radical. After radical formation it is able to perform multiple rounds of catalysis even under strict anaerobic conditions because of the regeneration of the enzyme radical. In this context it is important to remember that oxygen-independent CPO HemN is an enzyme generating the catalytically required radical via the homolytic cleavage of reduced S-adenosylmethionine (6).

To discriminate between a function of oxygen in radical formation or a possible single turnover function as electron acceptor purified recombinant HemF was analyzed under anaerobic conditions. HemF enzyme was anaerobically produced and purified to prevent the contact to molecular oxygen. As expected, this enzyme preparation failed to catalyze coprogen conversion under anaerobic conditions. This observation confirms the absolute oxygen requirement for HemF function. However, if HemF is exposed to oxygen during the recombinant protein production, but purified and tested anaerobically, a single turnover reaction can be monitored (Table I). These observations support an electron acceptor function for oxygen.

### Hydrogen Peroxide Development during E. coli HemF Catalysis

To follow oxygen reduction during coprogen oxidation, hydrogen peroxide formation was determined using a sensitive commercial detection system. After calibration of the test system hydrogen peroxide generation during HemF catalysis was observed (data not shown).

### E. coli HemF Is a Manganese-dependent CPO

A potential catalytic mechanism with the involvement of manganese was described for E. coli HemF. A, metal free catalysis.

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**Table III**

Kinetic parameters of produced HemF mutants

| HemF enzyme | Activity | $K_m$ | $V_{max}$ | Bound manganese |
|-------------|----------|-------|-----------|-----------------|
| Wild type   | 100      | 2.6   | 1.3       | +               |
| C167S       | 100      | 1.6   | 3         | NT             |
| H96L        | ND       | ND    | ND        | ND              |
| H106L       | ND       | ND    | ND        | ND              |
| H145L       | ND       | ND    | ND        | ND              |
| H175L       | ND       | ND    | ND        | ND              |
| Y135F       | 100      | 3.8   | 2.6       | NT              |
| Y160F       | 105      | 3.5   | 2.4       | NT              |
| Y170F       | 95       | 4.1   | 2.8       | NT              |
| Y213F       | 97       | 3     | 2.3       | NT              |
| Y240F       | 99       | 2     | 4         | NT              |
| Y276F       | 100      | 4.3   | 3         | NT              |
| W36L        | 98       | 4.3   | 3         | NT              |
| W123L       | 103      | 3.3   | 2.7       | NT              |
| W166L       | 100      | 3     | 2         | NT              |
| W274L       | ND       | ND    | ND        | +               |
| W286L       | 102      | 3.2   | 2.2       | NT              |
| W2124R      | 100      | 3.6   | 1         | NT              |
| G127V       | 105      | 2.1   | 1.7       | NT              |
| T132A       | 110      | 3     | 2         | NT              |
| P133A       | 98       | 3.5   | 2.5       | NT              |

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*HemF mutant enzymes were produced and purified as described under “Experimental Procedures.”

**Enzymatic assays were performed as described under “Experimental Procedures.”

**Manganese detection was performed via atomic absorption spectroscopy as described under “Experimental Procedures.”

**NT, not tested.

**ND, not detectable.

**Parentheses indicate varying manganese content (see text for details).
lyzed by *E. coli* HemF made the participation of metal ions very likely. Enzyme assays employing HemF pretreated with various metal chelators were performed. EGTA treatment resulted in a significant loss of CPO activity of HemF. Different metal ions in concentrations between 0.25 and 20 mM were added to the enzyme assay. Only the presence of manganese ions restored CPO activity with a maximum enzyme activity at a concentration of 10 mM. Other divalent metal ions including copper, iron, magnesium, cobalt, and nickel failed to reactivate HemF (Table II). In agreement with the observed metal dependence the enzyme was inhibited by low concentrations of heavy metal ions (Table II).

To investigate for a structural function of the bound manganese, gel permeation chromatography analysis was performed. Both native HemF and EGTA-treated enzyme showed a dimeric structure (data not shown). CD spectroscopy revealed no detectable difference between EGTA-treated and native HemF (data not shown). Therefore, a possible role of manganese for the maintenance of its dimeric structure was ruled out. Alternatively, participation of manganese in catalysis seemed to be possible.

**Identification of Histidine and Tryptophan Residues Essential for *E. coli* HemF Activity**—To initially analyze *E. coli* HemF for the participation of specific amino acid residues in catalysis and metal binding, chemical protein modification experiments were carried out. Previous studies using eukaryotic CPOs pointed toward a possible involvement of tyrosine (12) and histidine residues (8) in enzyme activity. In agreement with these observations, treatment of HemF with 2-hydroxy-5-nitrophenylboride, N-chlorosuccinimide, diethylpyrocarbonate, iodoacetamide, and tetranitromethane indicated the importance of histidine, tyrosine, and tryptophan residues for *E. coli* HemF activity (data not shown). To directly identify amino acid residues required for enzyme activity we mutated the codons for 6 highly conserved tyrosine residues in positions 135, 160, 170, 213, 240, and 276, for the conserved cysteine residue in position 167, for 6 tryptophan residues in positions 36, 123, 124, 166, 274, and 298, and for 4 histidine residues in positions 96, 106, 145, and 175 (Fig. 2). The purified recombinant *E. coli* HemFs bearing a mutated amino acid residue were first kinetically analyzed. Table III shows that mutation of each one of the four highly conserved histidine residues and of one tryptophan residue resulted in a complete loss of enzyme activity. Interestingly, the same histidine residues were mutated in the mouse enzyme (8), where only the mutation H158L, which corresponds to H106L in *E. coli*, abolished enzyme activity. None of the mutated tyrosine residues were found to be essential for enzyme activity. CD spectroscopy of the various mutated enzymes showed that none of the introduced mutations caused a significant change in protein structure (data not shown). A short conserved sequence motif (GGGXXTP) was also found in oxygen-independent CPOs (HemN) and some other nucleotide-binding enzymes (6, 7). We introduced four mutations of this sequence motif, each introducing a single amino acid residue exchange. However, none of these amino acid exchanges influenced enzyme activity (Table III, last four rows). In agreement, addition of several different nucleotides to the oxygen-dependent CPO activity assay did not change enzyme activity (Table II).

**Histidine Residues Potentially Involved in Manganese and Porphyrin Binding**—The essential histidine residues could be involved in metal binding or in other important catalytic processes. Interestingly, the UV-visible spectra of HemF-H96L, HemF-H106L, and HemF-H145L were identical to the spectrum of the wild type enzyme, whereas the HemF mutant H175L showed no absorbance related to bound porphyrins (Fig. 5). This observation might indicate a possible role of histidine 175 in substrate or product binding.

The wild type enzyme and various inactive HemF mutant enzymes were subjected to metal analysis using atomic absorption spectroscopy. No significant amounts of iron or copper were detected. Because of the incomplete manganese insertion into recombinant wild type HemF, indicated by the strong stimulation of enzyme activity by the addition of manganese to the purified protein, only 0.2 to 0.6 mol of manganese/mol of HemF were measured. In contrast to that, mutant HemF H106L did not contain detectable amounts of manganese, whereas H96L showed a significantly reduced manganese content. However, the manganese content of H145L and H175L varied between different preparations within a range of 0.04 to 0.4 mol of manganese/mol of subunit (H145L) and 0.005 to 0.5 mol of manganese/mol of subunit (H175L). This observation points toward a potential, however, not essential role of this residues in metal chelation. Nevertheless, all investigated mutations of histidine residues lead to a complete inactivation of HemF. The potential double function of His-175 in substrate/product binding and manganese coordination is subject to ongoing work.

**The Role of Manganese in *E. coli* HemF Activity**—In nature manganese is found to coordinate and activate oxygen. One example for such a manganese containing enzyme is oxalate oxidase, which converts oxalic acid using oxygen to CO₂ and H₂O₂ (25). Biochemical and spectroscopic studies demonstrated that barley oxalate oxidase utilizes a mononuclear manganese center for catalysis (26, 27). One proposed mechanism for the barley enzyme includes the involvement of a Mn(III)-superoxo form in catalysis (28). In light of the radical enzyme mechanism elucidated for the oxygen-independent CPO HemN a possible role of HemF-bound manganese could lay in the binding of O₂ to produce a Mn(III)-superoxo species. The formed radical leads to the abstraction of a hydrogen atom from the β-C atom of the propionate side chain of the substrate and the formation of a substrate radical (Fig. 6A). During the final formation of the vinyl group with the elimination of CO₂, oxygen could function as electron acceptor leading to hydrogen peroxide formation. Alternatively, a purely structural but essential role of manganese without any participation in the enzymatic mechanism is a second possibility (Fig. 6B). To our knowledge currently there is no proven model available for an oxygen-dependent enzyme mechanism without cofactor or metal participation. Nevertheless, at this state we cannot experimentally distinguish between these two possible roles of manganese in HemF function.

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