In bacteria the oxygen-independent coproporphyrinogen-III oxidase catalyzes the oxygen-independent conversion of coproporphyrinogen-III to protoporphyrinogen-IX. The Escherichia coli hemN gene encoding a putative part of this enzyme was overexpressed in E. coli. Anaerobically purified HemN is a monomeric protein with a native $M_r = 52,000 \pm 5,000$. A newly established anaerobic enzyme assay was used to demonstrate for the first time in vitro coproporphyrinogen-III oxidase activity for recombinant purified HemN. The enzyme requires S-adenosyl-$L$-methionine (SAM), NAD(P)H, and additional cytoplasmatic components for catalysis. An oxygen-sensitive iron-sulfur cluster was identified by absorption spectroscopy and iron analysis. Cysteine residues Cys$^{56}$, Cys$^{66}$, and Cys$^{88}$, which are part of the conserved CXXXCXXC motif found in all HemN proteins, are essential for iron-sulfur cluster formation and enzyme function. Completely conserved residues Tyr$^{56}$ and His$^{56}$, localized closely to the cysteine-rich motif, were found to be important for iron-sulfur cluster integrity. Mutation of Gly$^{111}$ and Gly$^{113}$, which are part of the potential GGTP S-adenosyl-$L$-methionine binding motif, completely abolished enzymatic function. Observed functional properties in combination with a recently published computer-based enzyme classification (Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F., and Miller, N. E. (2001) Nucleic Acids Res. 29, 1097–1106) identifies HemN as “Radical SAM enzyme.” An appropriate enzymatic mechanism is suggested.

The biosynthesis of the tetrapyrrole ring of hemes and chlorophylls requires the oxidative decarboxylation of coproporphyrinogen-III to form protoporphyrinogen-IX catalyzed by coproporphyrinogen-III oxidases (CPOs, EC 1.3.3.3) (1–4). During this reaction the propionate side chains of ring A and B of coproporphyrinogen-III are consecutively converted to the corresponding vinyl groups (see Fig. 1A) (5). Under aerobic conditions molecular oxygen serves as one possible electron acceptor for catalysis. Based on these bioinformatic predictions a novel appropriate enzymatic mechanism is suggested.

Oxygen-independent Coproporphyrinogen-III Oxidase HemN from Escherichia coli*

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§ The abbreviations used are: CPO, coproporphyrinogen-III oxidase; HPLC, high performance liquid chromatography; SAM, S-adenosyl-$L$-methionine.
cloning of the E. coli hemN gene into an expression vector, its overexpression, and the purification of the recombinant protein to apparent homogeneity. An oxygen-independent CPO assay was established, proving for the first time that HemN is indeed an oxygen-independent CPO. The cofactor requirements for the catalytic process were elucidated. Site-directed mutagenesis was used to investigate the role of the conserved cysteine residues (Cys62, Cys66, Cys69, and Cys71) for [4Fe-4S] cluster formation and the importance of other conserved amino acid residues. A catalytic mechanism for the oxygen-independent CPO was proposed.

**Experimental Procedures**

**Construction of the E. coli hemN Expression Vector pET3ahemN—** A 1371-bp pair fragment encoding all 457 amino acid residues of E. coli HemN was amplified by PCR using primer ND1ET3 (5'-TAGCCGCCAATTGCACTGGGTGCGTAACGCCGACG) and primer BH13A (5'-CATATGCCATCGAGCGGGATCCACTATTCTCTCTCTACGTACTTATCCCGTTCTGCC). The resulting PCR fragment was digested with NdeI and BamHI (recognition sequences underlined in the primer sequences) and ligated into the appropriately digested vector pET3a to generate pET3ahemN.

**Site-directed Mutagenesis of E. coli hemN—** To exchange amino acid residues of E. coli HemN, the QuikChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) was used according to the manufacturer’s instructions. Oligonucleotides carrying nucleotide exchanges compared with the hemN sequence in the underlined positions were used to generate the mutants indicated: for Y56F, GCGTCCATTGCACTGGGTGCGTAACGCCGACG; for C66S, CCGTTCTCTCTCTACGTACTTATCCCGTTCTGCC; for C62S, CGTACATATCCCGTTCTCTCTCTACTTACGTAACGCCG; and for F68L, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG. All mutated genes were used to generate the mutants indicated: for Y56F, GCGTCCATTGCACTGGGTGCGTAACGCCGACG; for C66S, CCGTTCTCTCTCTACGTACTTATCCCGTTCTGCC; for C62S, CGTACATATCCCGTTCTCTCTCTACTTACGTAACGCCG; and for F68L, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG. All mutated genes were subjected to complete DNA sequence determination.

**Overexpression of E. coli hemN and Purification of HemN—** E. coli BL21(DE3) carrying wild type or mutant pET3ahemN was grown under strictly anaerobic conditions at 37 °C in Spizizen’s minimal medium (27) containing 10 mM NaNO3, 2 mM Fe(III)-citrate, and 100 μg/ml ampicillin. When cultures reached an A578 of 0.35, isopropyl-β-D-thiogalacto-pyranoside was added to a final concentration of 50 μM. The cells were further cultivated for 1 h at 30 °C after induction and finally stored at 4 °C for about 15 h to allow iron-sulfur cluster formation. Subsequently the cells were anaerobically harvested by centrifugation. All further steps were carried out under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, MI). All solutions used were saturated with N2 prior to use. The bacterial cell pellet was resuspended in 10 ml of buffer A (50 mM Tris-HCl (pH 8.0), 0.1% (v/v) Triton X-100, 3 mM dithiothreitol). Cells were disrupted by a single passage through a French press at 1500 p.s.i. Cell debris and the insoluble protein fraction were removed by centrifugation for 60 min at 175,000 × g at 4 °C. The resulting supernatant was loaded onto a 30-ml Blue Sepharose XK26 column (Amersham Biosciences), which had previously been equilibrated with buffer A. The column was washed with 200 ml of buffer B to remove unbound proteins. Bound proteins were eluted using a 120-ml linear gradient of 0–1 M NaCl in buffer B at a flow rate of 1 ml/min. Recombinant HemN eluted at ~600–900 mM NaCl. Fractions containing HemN were identified using SDS-polyacrylamide gel electrophoresis, pooled, and concentrated by ultrafiltration (Amicon, Millipore GmbH, Eschborn, Germany).

**Preparation of E. coli Cell-free Extract (S250) Required to Restore HemN Activity—** E. coli strains were grown anaerobically at 37 °C in Terrific Broth medium (28) to an A578 of 0.7. Cells were harvested by centrifugation. All further steps were carried out under strictly anaerobic conditions.

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**FIG. 1.** Oxidative decarboxylation of coproporphyrinogen-III to protoporphyrinogen-IX and amino acid sequence alignments of potential Radical SAM enzymes. A. The propionate side chains of coproporphyrinogen-III ring A and B are successively converted to the corresponding vinyl groups (5). B. Alignment of HemN proteins from indicated different bacteria with members of the Radical SAM protein family. The aligned proteins are HemN and Bacillus halodurans HemZ, Thauera aromatica benzosulinate synthase-activating enzyme (BssD), E. coli bibd synthase (BioB), E. coli lipoate synthase (LipA), Clostridium subterminale lysine 2,3-aminomutase (LAM), E. coli pyruvate formate-lyase-activating enzyme (PFL-AE), E. coli anaerobic ribonucleotide reductase-activating enzyme (ARNR-AE), and B. halodurans spore photoprotein lyase (SP lyase). The iron-sulfur cluster-coordinating CXX(CX)XXX motif of HemN, HemZ, and the Radical SAM proteins is shown. The conserved cysteine residues are marked by shaded boxes. In the amino acid sequences of the Radical SAM proteins including HemZ (24). Other mutated residues (Tyr56, His58, and Phe68) of HemN were used to generate the mutants indicated: for Y56F, GCGTCCATTGCACTGGGTGCGTAACGCCGACG; for C66S, CCGTTCTCTCTCTACGTACTTATCCCGTTCTGCC; for C62S, CGTACATATCCCGTTCTCTCTCTACTTACGTAACGCCG; and for F68L, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG. All mutated genes were subjected to complete DNA sequence determination.

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**Tetrapyrrole Biosynthesis and HemN**

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**experimental procedures**

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**Site-directed Mutagenesis of E. coli hemN—** To exchange amino acid residues of E. coli HemN, the QuikChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) was used according to the manufacturer’s instructions. Oligonucleotides carrying nucleotide exchanges compared with the hemN sequence in the underlined positions were used to generate the mutants indicated: for Y56F, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG; for H58L, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG; for C66S, CGTACATATCCCGTTCTCTCTCTACTTACGTAACGCCG; and for F68L, GCCATATTCCCGTTCTCTCTCTACTTACGTAACGCCG.
conditions in an anaerobic chamber (Coy Laboratories). The bacterial cell pellet was resuspended in buffer B (50 mM Tris-HCl (pH 8.0), 3 mM dithiothreitol), and cells were disrupted by a single passage through a French press at 1500 p.s.i. Cell debris and all insoluble components were removed by centrifugation for 60 min at 250,000 × g at 4 °C. The resulting protein solution (20 mg/ml) was stored at −20 °C.

**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.

**N-terminal Amino Acid Sequence Determination**—The N-terminal amino acid sequence of the purified enzyme was determined by Edman degradation.

Electrospray Ionization Mass Spectrometry—Electrospray ionization mass spectrometry data were collected using a Finnigan Mat TSQ 7000 spectrometer. The analysis was carried out as described before (29).

**Iron Determination Methods**—Protein-bound iron was determined colorimetrically with o-phenanthroline after acid denaturation of purified HemN (31). Additionally the iron content of HemN was determined by flame atomic absorption spectroscopy using a PE 3100 (PerkinElmer Instruments, Uberlingen, Germany). The iron standards were purchased from Merck.

UV-Visible Light Absorption Spectroscopy—UV-visible light spectra of purified recombinant HemN were recorded using a Lambda 2 spectrophotometer (PerkinElmer Instruments) under strict anaerobic conditions.

**Coproporphyrinogen-III Oxidase Assay Using Recombinant HemN**—The coproporphyrinogen-III oxidase assay was carried out under strict anaerobic conditions. Reaction components were mixed in an anaerobic chamber. All solutions used were saturated with N2 prior to use. The standard assay mixture contained, in a total volume of 300 μl, 50 μg of recombinant HemN (3.2 μM), 1.32 mg of protein of an E. coli cell-free extract (S250), 0.5 mM SAM, 0.5 mM NADH, and 0.3% (v/v) Triton X-100 in buffer A. Further additions are indicated under "Results and Discussion" and in Table I. Coproporphyrinogen-III was prepared by reduction of coproporphyrin-III (Porphyrin Products Inc., Logan, UT) using sodium amalgam (32). Dithiothreitol (50 mM) was added to the coproporphyrinogen-III solution, which was subsequently adjusted to pH 8.0 with 20% phosphoric acid. The coproporphyrinogen-III solutions were stored at −65°C. The reaction was started by addition of coproporphyrinogen-III (20 μM) to the assay mixture. After incubation at 37 °C for 1 h in the dark, the reaction was stopped and formed proporphyrinogen-IX was oxidized for 15 min to protoporphyrin-IX by addition of 30% H2O2. The amount of enzymatically formed protoporphyrinogen-IX was determined by fluorimetric detection of its oxidized form protoporphyrinogen-IX using a PE LS50B luminescence spectrometer (PerkinElmer Instruments) with an excitation wavelength of 409 nm and an emission wavelength of 630 nm and in parallel using photometric diode array analysis for 200–650 nm. The HPLC system was the Jasco 1500 series (Jasco, Gross-Umstadt, Germany). Coproporphyrin-III and proporphyrin-IX (Sigma) were used as porphyrin standards.

**Results and Discussion**

**Production of Recombinant E. coli HemN**—The E. coli hemN gene was expressed using a TT RNA polymerase-driven system. Recombinant HemN was purified to apparent homogeneity using a single chromatographic step on Blue Sepharose. Production of recombinant HemN was confirmed by SDS-PAGE and mass spectrometry. The native molecular mass of recombinant HemN was determined using gel permeation chromatography (data not shown). A relative native molecular weight of 52,000 ± 5,000 for HemN was deduced from both experiments. This is in good correlation with the calculated molecular mass of 52,734 Da and identifies E. coli HemN as a monomeric protein (data not shown).

**Classification of HemN as SAM-dependent Radical Enzyme**—A recently published computational investigation of iron-sulfur cluster-constituting amino acid motifs (Fig. 1B) placed HemN in a class of SAM-dependent radical enzymes.
HemN Carries Oxygen-independent CPO Activity—To test this hypothesis a new anaerobic CPO assay was established. For that purpose purified recombinant HemN was incubated under strict anaerobic conditions in the presence of various cofactors and an E. coli cytoplasmic cell-free extract (S250) for 1 h in the dark at 37 °C with coproporphyrinogen-III as substrate. Formed protoporphyrinogen-IX and remaining coproporphyrinogen-III substrate were detected after oxidation to the appropriate porphyrins using H$_2$O$_2$ via fluorescence spectroscopy and HPLC (Fig. 3, B and C). Maximal oxygen-independent CPO activity for recombinant E. coli HemN was observed in the presence of 100 μM SAM, 0.5 mM NADH, and unidentified cytoplasmic components (Table I). Almost complete conversion of coproporphyrinogen-III into protoporphyrinogen-IX was detected using fluorescence spectroscopy (Fig. 3B). The E. coli S250 used carried only insignificantly low CPO activity in the absence of recombinant HemN under these conditions. HPLC analysis of the reaction reconfirmed the almost complete conversion of coproporphyrinogen-III into protoporphyrinogen-IX by recombinant HemN (Fig. 3C). As described before (10), using cell-free extracts of various bacteria the addition of ATP, l-Met, and Mg$^{2+}$ to the assay partially substituted for SAM requirement, indicating SAM synthesis by the cytoplasmic extract. Several different nicotinamide cofactors were utilized by the enzymatic system with NADH yielding the highest enzyme activity (Table I). One explanation for the observed broad spectrum of utilized nicotinamide cofactors is their interconversion by enzymes present in the cytoplasmic fraction. The reduced nicotinamide cofactors could serve as substrate for an oxidoreductase for the generation of an electron donor like a flavodoxin. The addition of an E. coli cell-free cytoplasmic extract was absolutely required to obtain CPO activity. We assume that the major role of the essential E. coli fraction (S250) in HemN activity was its assumed electron donor and electron acceptor function (see model in Fig. 4). In agreement with this assumption phenol extraction and gel permeation chromatography of the cytoplasmic fraction identified proteinogenic components with a $M_r$ larger than 10,000 required for the efficient reconstitution of HemN-mediated CPO activity (Table I). The proteins of an electron donor system, like NADPH-dependent flavodoxin oxidoreductase and the flavodoxin, could represent these high molecular mass components. The assay mixture always contained a 0.3% (v/v) concentration of the detergent Triton X-100. Without detergent addition no CPO activity was detectable (Table I). As observed for other CPOs E. coli HemN might be membrane-associated in vivo (2), and sufficient solubilization is required for enzyme activity.

E. coli HemN Contains an Oxygen-sensitive Iron-Sulfur Cluster—Concentrated HemN solution was yellow-brown in color. An UV-visible light absorption spectrum recorded under anaerobic conditions revealed an increase of absorption at 320 nm and a major absorption peak around 410 nm (Fig. 2B). The observed absorption was continuously lost over a period of 24 h in the presence of oxygen. These features are typical for many proteins containing [4Fe-4S] clusters. To determine whether the spectroscopic features of E. coli HemN were due to the presence of an iron-sulfur cluster, the iron content of the protein was analyzed. Analysis using atomic absorption spectroscopy and a chemical determination method showed the presence of enzyme-bound iron that ranged from 1.3 to 3 mol of iron/mol of HemN monomer. These results indicated the presence of reasonable amounts of iron in HemN. However, due to the high level production of recombinant HemN in E. coli (up to 40% of the total cellular protein) only incomplete iron-sulfur cluster formation was achieved leading to the observed values.
and other SAM-dependent radical enzymes (Fig. 1)

CPO activity has been described before (14). In addition of iron chelators (EDTA and DTPA) came from the strong inhibition of enzyme activity by the iron-sulfur clusters are often reconstituted in recombinant enzymes were produced in E. coli BL21(DE3) under conditions of iron coordination, it is important for HemN activity all four cysteine residues Cys62, Cys66, and Cys69 of HemN are not essential for iron coordination, it is important for HemN activity. The concentration of iron was determined with the addition of cell-free extract (S250) and the cofactors SAM and NADH was measured, low background activity (below 10%) resulting from S250 addition was subtracted, the obtained value was set to 100%, and all other measured CPO activities were related to that.

Requirements for HemN catalysis

| HemN<sup>a</sup> | Cell-free extract<sup>b</sup> (S250) | SAM | Nicotinamide cofactor | 0.3% (v/v) Triton X-100 | Other additions<sup>c</sup> | CPO activity<sup>d</sup> |
|-----------------|-------------------------------|-----|------------------------|---------------------------|-------------------------|---------------------|
| +               | +                             | 0.5 mM NADH | +                      | –                         | ATP, L-Met, Mg<sup>2+</sup> | 21                  |
| –               | +                             | 1.7 μM NADH | +                      | –                         | –                       | 7                   |
| +               | +                             | 3 μM NADH | +                      | –                         | –                       | 10                  |
| +               | +                             | 5 μM NADH | +                      | –                         | –                       | 14                  |
| +               | +                             | 7 μM NADH | +                      | –                         | –                       | 16                  |
| +               | +                             | 10 μM NADH | +                      | –                         | –                       | 27                  |
| +               | +                             | 20 μM NADH | +                      | –                         | –                       | 41                  |
| +               | +                             | 40 μM NADH | +                      | –                         | –                       | 62                  |
| +               | +                             | 100 μM NADH | +                      | –                         | –                       | 100                 |
| +               | +                             | 500 μM NADH | +                      | –                         | –                       | 100                 |
| +               | +                             | 0.5 mM NADPH | +                      | –                         | –                       | 58                  |
| +               | +                             | 0.5 mM NADH | +                      | –                         | –                       | 27                  |
| +               | +                             | 0.5 mM NAD<sup>–</sup> | +                      | –                         | –                       | 42                  |
| +               | +                             | 0.5 mM NADH | +                      | –                         | EDTA                     | 3                   |
| +               | +                             | 0.5 mM NADH | +                      | –                         | o-Phenanthroline         | ND                  |

<sup>a</sup> Purified, recombinant HemN (3.2 μM) was used in the assay mixtures.

<sup>b</sup> E. coli cell-free extract (1.32 mg of protein) prepared as described under “Experimental Procedures” was used in the assay mixtures.

<sup>c</sup> Cofactors and other additions were added to the assay mixture when indicated at the following concentrations: 5 mM MgSO<sub>4</sub>, 4 mM ATP, 1 mM L-Met, 2 mM EDTA, 2 mM o-phenanthroline.

<sup>d</sup> HemN CPO activity with the addition of cell-free extract (S250) and the cofactors SAM and NADH was measured, low background activity (below 10%) resulting from S250 addition was subtracted, the obtained value was set to 100%, and all other measured CPO activities were related to that.

<sup>e</sup> ND, not detectable.

<sup>f</sup> E. coli S250 was separated into a low and high molecular mass fraction by ultrafiltration through an Amicon PBGC membrane (relative molecular mass exclusion of 10,000) (Amicon, Millipore GmbH).

Appropriate variations in enzyme activity were observed. It is a commonly observed problem that iron incorporation into recombinant Fe-S proteins in vivo is incomplete (35). Therefore, the iron-sulfur clusters are often reconstituted in vitro after purification of the apoproteins. However, we failed to improve the iron-sulfur cluster content of HemN by chemical methods. Further strong evidence for the presence of an iron-sulfur cluster came from the strong inhibition of enzyme activity by the addition of iron chelators (EDTA and o-phenanthroline) to the test system (Table I). Iron-stimulated oxygen-independent CPO activity has been described before (14).

### Cysteine Residues Cys<sup>62</sup>, Cys<sup>66</sup>, and Cys<sup>69</sup> Are Involved in Iron-Sulfur Cluster Formation—Amino acid alignments identified four clustered cysteine residues conserved in all HemN proteins (Fig. 1B). The fourth residue Cys<sup>71</sup> is missing in HemZ and other SAM-dependent radical enzymes (Fig. 1B). To determine which of the four conserved cysteine residues (C62S, C66S, C69S, and C71S) is involved in iron-sulfur cluster formation and important for HemN activity all four cysteine residues were individually changed to serine residues. The mutated enzymes were produced in E. coli BL21(DE3) under conditions identical to the wild type enzyme and purified to homogeneity (Fig. 2A). The concentrated protein solutions of mutant HemN proteins C62S, C66S, and C69S were colorless, while the solution of mutant enzyme C71S was yellow-brown in color. The absorption spectra of the mutant enzymes were recorded under anaerobic conditions. For the mutants C62S, C66S, and C69S no absorption peak at 410 nm and no shoulder at 320 nm were detected in accordance with the colorless protein solutions. In Fig. 2B, the C62S absorption spectrum is shown in comparison to the spectrum of the wild type enzyme. The spectra of C66S and C69S were nearly identical (data not shown). Mutant HemN C71S revealed a distinct absorption maximum at 410 nm with a shoulder at 320 nm comparable to the wild type HemN absorption spectrum (Fig. 2B). Iron content analysis was performed for the mutant HemN enzymes C62S, C66S, C69S, and C71S. No significant amounts of iron were detected using atomic absorption spectroscopy and chemical iron determination for C62S, C66S, and C69S. For C71S 1.1–3 mol of iron/mol of protein was determined. These results, in agreement with the absorption spectra of the various HemN mutant enzymes, indicated that C62S, C66S, and C69S did not possess any detectable Fe-S cluster, while the mutant enzyme C71S still carried the cluster. Therefore, only the cysteine residues Cys<sup>62</sup>, Cys<sup>66</sup>, and Cys<sup>69</sup> of E. coli HemN are essential for iron coordination, whereas Cys<sup>71</sup> is not. These results are in accordance with the proposed sequence motif CXXCCXXC that HemN shares with the proteins of the Radical SAM family including HemZ (24). The CPO activity of the four mutant proteins was tested (Table II). All four HemN mutants C62S, C66S, C69S, and C71S had lost their CPO activity. The loss of CPO activity for the HemN mutants C62S, C66S, and C69S was due to the missing Fe-S cluster. These observations underscore the importance of the HemN Fe-S cluster for the catalytic process. Although the fourth conserved cysteine residue Cys<sup>71</sup> of HemN is not essential for iron coordination, it is important
FIG. 4. Postulated enzymatic mechanism for HemN. A 5'-deoxyadenosyl radical is formed by the homoltyc cleavage of SAM. The electron needed for this cleavage is obtained from the [4Fe-4S] cluster. The 5'-deoxyadenosyl radical abstracts a hydrogen atom from the propionate side chain of the substrate to form 5'-deoxyadenosine and a substrate radical. The vinyl group of the CPO reaction product is formed by elimination of CO₂. The vinyl group of the CPO reaction product is formed by elimination of CO₂. For a new reaction cycle the Fe-S cluster requires reduction. For that purpose NAD(P)H-flavodoxin oxidoreductase and flavodoxin could serve as electron donor. Identified components of the model are boxed. Components potentially located in the E. coli S250 used are shown in brackets. ox, oxidized form; red, reduced form.

Table II: CPO activity and iron contents of the HemN mutants C62S, C66S, C69S, C71S, Y56F, H58L, F68L, G111V/G113V

| HemN | CPO activity | Fe-S cluster |
|------|--------------|--------------|
| Wild type | 100 | + |
| C62S | ND | – |
| C66S | ND | – |
| C69S | ND | – |
| C71S | ND | + |
| Y56F | 45 | (+)* |
| H58L | ND | – |
| F68L | 89 | – |
| G111V/G113V | ND | (+)* |

* HemN mutant enzymes were produced and purified as described under "Experimental Procedures.”
The CPO activity assay mixtures contained 3.2 μM HemN (wild type or mutant), E. coli cell-free extract (1.32 mg of protein), 0.5 mM SAM, 0.5 mM NADH, 0.3% (v/v) Triton X-100. The CPO activity obtained for wild type HemN minus the CPO activity of the cell-free extract alone was set to 100%, and the other measured CPO activities were related to that.

UV-visible light absorption spectra were recorded under anaerobic conditions. The presence of a Fe-S cluster was indicated by an absorption maximum at 410 nm. In addition iron determinations were performed.

ND, not detectable.

* Significantly reduced iron-sulfur cluster formation was observed.

Amino Acid Residues Tyr56, His58, Gly111, and Gly113 Are Important for Catalysis—Amino acid residues Tyr56 and His58 are adjacent to the iron-coordinating cysteine-rich motif. They are highly conserved in HemN and HemZ proteins. In the light of a potential radical mechanism one can speculate that Tyr56 is involved in catalysis by carrying or stabilizing an enzyme-localized radical. His58 could play a role as a potential fourth ligand for the iron-sulfur cluster. The highly conserved amino acid residue Phe68 lies within the YXHXPFCXXXCFP motif. Interestingly, a conserved aromatic residue (Tyr, Phe, or Trp) adjacent to the third cysteine occurs in all Radical SAM proteins (24). This aromatic residue (Phe68 for E. coli HemN) might function to lower the midpoint potential of the iron-sulfur cluster (38). Finally, the glycine-rich sequence motif GGPTP115 was proposed to be a potential SAM binding site (24).

To test the function of these residues Tyr56, His58, Phe68, and Gly111 and Gly113 were exchanged by site-directed mutagenesis. The mutant enzymes Y56F, H58L, F68L, and G111V/G113V were produced and purified under conditions identical to the wild type enzyme (Fig. 4).

The results of our characterization of the mutant enzymes are shown in Table II. Mutation of residue His58, close to the cysteine residues constituting the iron-sulfur cluster, totally abolished enzyme activity. No iron-sulfur cluster was detected for mutant H58L. Therefore, His58 might be involved in iron-sulfur cluster formation in providing the fourth ligand besides the three cysteine residues in positions 62, 66, and 69. Mutant Y56F showed only reduced enzyme activity compared with the wild type enzyme making a central function of residue Tyr56 in enzyme radical formation unlikely. The observed partial loss of the iron-sulfur cluster by exchange of Tyr56 indicated a potential role of Tyr56 in cluster stabilization. Clearly, replacement of Tyr56 by Phe partially compensated for potential structural or catalytic functions of this conserved residue. Interestingly, mutant F68L was found to be as active as the wild type HemN. Double mutation of the glycine-rich motif (G111V/G113V) potentially involved in SAM binding totally abolished CPO activity. Additionally, a significant destabilization and partial loss of the iron-sulfur cluster was observed indicating close structural relation between the iron-sulfur cluster and the putative SAM binding site. Structural close contact of SAM to the iron-sulfur cluster seems to be important for radical formation (37, 38).

An Enzymatic Mechanism for E. coli HemN: A Proposal—Based on the assumption that HemN belongs to the Radical SAM protein family and considering the mechanisms that have been put forward for other members of the family (20, 25, 26, 34, 39, 40), we postulate a radical mechanism for the oxidative side chain carboxylation of coproporphyrinogen-III catalyzed by HemN (Fig. 4). The first step in our mechanism is the reduction of the [4Fe-4S]²⁻ to a [4Fe-4S]³⁺ cluster by one electron followed by the homoltyc cleavage of SAM to methionine and a 5'-deoxyadenosyl radical. Thereby the Fe-S cluster is oxidized back to the [4Fe-4S]²⁻ form. The 5'-deoxyadenosyl radical abstracts a hydrogen atom from the β-C atom of the propionate side chain of the substrate and generates the corresponding substrate radical. Stereospecific hydrogen abstraction from the β-C atom of the substrate radical. The vinyl group of the CPO reaction product is formed by elimination of CO₂. For a new reaction cycle the Fe-S cluster requires reduction. For that purpose NAD(P)H-flavodoxin oxidoreductase and flavodoxin could serve as electron donor. Identified components of the model are boxed. Components potentially located in the E. coli S250 used are shown in brackets. ox, oxidized form; red, reduced form.
the substrate propionate group during CPO catalysis has been reported previously (11). During the final step the vinyl group of protoporphyrinogen-IX is formed, and CO2 is released. This step requires an electron acceptor for the remaining electron of the substrate.

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