The Substrate Radical of Escherichia coli Oxygen-independent Coproporphyrinogen III Oxidase HemN*

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During porphyrin biosynthesis the oxygen-independent coproporphyrinogen III oxidase (HemN) catalyzes the oxidative decarboxylation of the propionate side chains of rings A and B of coproporphyrinogen III to form protoporphyrinogen IX. The enzyme utilizes a 5′-deoxyadenosyl radical to initiate the decarboxylation reaction, and it has been proposed that this occurs by stereo-specific abstraction of the pro-S-hydrogen atom at the β-position of the propionate side chains leading to a substrate radical. Here we provide EPR-spectroscopic evidence for intermediacy of the latter radical by observation of an organic radical EPR signal in reduced HemN upon addition of S-adenosyl-L-methionine and the substrate coproporphyrinogen III. This signal (gav = 2.0029) shows a complex pattern of well resolved hyperfine splittings from at least five different hydrogen atoms. The radical was characterized using regiospecifically labeled (deuterium or 15N) coproporphyrinogen III molecules. They had been generated from a multienzyme mixture and served as efficient substrates. Reaction of HemN with coproporphyrinogen III, perdeuterated except for the methyl groups, led to the complete loss of resolved proton hyperfine splittings. Substrates in which the hydrogens at both α- and β-positions, or only at the β-positions of the propionate side chains, or those of the methylene bridges, were deuterated showed that there is coupling with hydrogens at the α-, β-, and methylene bridge positions. Deuterium or 15N labeling of the pyrrole nitrogens without labeling the side chains only led to a slight sharpening of the radical signal. Together, these observations clearly identified the radical signal as substrate-derived and indicated that, upon abstraction of the pro-S-hydrogen atom at the β-position of the propionate side chain by the 5′-deoxyadenosyl radical, a comparatively stable delocalized substrate radical intermediate is formed in the absence of electron acceptors. The observed hyperfine constants and g values show that this coproporphyrinogenyl radical is allylic and encompasses carbon atoms 3′, 3, and 4.

Modified tetrapyroles such as hemes and chlorophylls play important roles in a range of essential life processes from respiration to photosynthesis. Their underlying molecular architecture is reflected in a shared biosynthetic pathway that requires the coordinated activity of a large number of highly diverse enzymes (1–3). During porphyrin formation two structurally unrelated coproporphyrinogen III oxidases catalyze the oxidative decarboxylation of the propionate side chains on pyrrole rings A and B of the macrocycle to the corresponding vinyl groups (Scheme 1a) (4). The oxygen-dependent enzyme, HemF, found in eukaryotes and some bacteria, uses molecular oxygen as an electron acceptor during this process (5). For oxygen-independent coproporphyrinogen formation most bacteria carry the oxygen-independent enzyme HemN. In Escherichia coli, HemN is a monomeric protein that contains an oxygen-sensitive [4Fe-4S] cluster (6, 7). The enzyme belongs to the Radical SAM (S-adenosyl-L-methionine) protein family, an emerging enzyme class with over 600 putative members (8). All Radical SAM enzymes use a reduced [4Fe-4S] cluster for the homolytic cleavage of SAM (Scheme 1b) (9–11). In HemN the resulting 5′-deoxyadenosyl radical is proposed to abstract the pro-S-hydrogen atom at the β-carbon (18) of the substrate propionate side chain (Scheme 1c) (6). This process should result in the formation of 5′-deoxyadenosine and a substrate radical. To complete the reaction the C5′-COO− bond is cleaved, CO2 is released, and the vinyl group of the reaction product is formed. During this process an electron acceptor is required for the removal of the remaining electron of the substrate radical.

Other well known members of this family include the activases of anaerobic ribonucleotide reductase (anaerobic ribonucleotide reductase activating enzyme) and pyruvate formate-lyase (pyruvate formate-lyase activating enzyme) as well as lysine 2,3-aminomutase (LAM), biotin synthase, lipoate synthase, and the molybdenum cofactor precursor Z synthase. In these enzymes the 5′-deoxyadenosyl radical abstracts a hydrogen atom either from an enzyme to generate a catalytically active glycol radical, as in anaerobic ribonucleotide reductase and pyruvate formate-lyase (12, 13), or from a substrate molecule to generate the corresponding substrate radical as in LAM (14). In some cases like LAM and spore photoproduct lyase SAM is reversibly cleaved and regenerated after each catalytic cycle (10, 15). In other cases like biotin synthase, pyruvate formate-lyase activating enzyme and HemN SAM is irreversibly cleaved and should be considered as a cosubstrate rather than a

8 This work was supported by grants of the Deutsche Forschungsgemeinschaft (priority program Radicals in Enzymatic Catalysis) and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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3 The abbreviations used are: SAM, S-adenosyl-L-methionine; ALA, 5-aminolevulinic acid; HPLC, high performance liquid chromatography; LAM, lysine 2,3-aminomutase; KOD, potassium deuterioxide; mT, millilitress.
HemN catalysis does not require pyridoxal 5'-phosphate. HemN utilizes two SAM molecules that are irreversibly cleaved into a covalently bound macromolecular, possibly electron-delocalizing substrate. For this purpose, HemN catalyzes the decarboxylation reactions of the propionate side chains on rings A and B of coproporphyrinogen III to yield the vinyl groups of protoporphyrinogen IX. A, Radical SAM enzymes use a reduced [4Fe-4S] cluster for the homolytic cleavage of SAM to yield methionine and a catalytically active 5'-deoxyadenosyl radical. B, in the HemN reaction, the adenosyl radical is proposed to abstract a hydrogen atom from the β-carbon of the substrate propionate side chain resulting in the formation of a substrate radical. Release of CO2 and uptake of the remaining electron by an external electron acceptor leads to product formation. R = tetrapyrrole.

**MATERIALS AND METHODS**

**Materials**—All chemicals were purchased from either Sigma-Aldrich or Merck Eurolab (Darmstadt, Germany). Coproporphyrin III was purchased from Paesel and Lorei GmbH & Co. (Hanau, Germany). 5-[15N]Aminolevulinic acid hydrochloride (99 atom % 15N) and [1H]succinic anhydride (99 atom % D) were obtained from C/D/N Isotopes (Quebec, Canada). Deuterium chloride (35 weight. %, 99 atom % D), D2O (99.9 atom % D), and KOD (40 weight % solution in D2O, 98 atom % D) were purchased from Aldrich.

**Enzymes**—Recombinant *E. coli* HemN was produced and purified anaerobically as previously described (6).

Recombinant *Rhodobacter sphaeroides* ALA synthase (HemA) was produced and purified as follows. The *R. sphaeroides* hemA gene was amplified using PCR and cloned into the EcoRI/Sall sites of the expression vector pGEX-6P-1 (Amersham Biosciences) to produce pGEX-hemA. *E. coli* BL21(DE3) CodonPlus carrying pGEX-hemA was grown at 37 °C in LB medium containing 34 μg/ml chloramphenicol and 100 μg/ml ampicillin. When cultures reached an A600 of 0.4 isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 500 μM, and the cells were further cultivated overnight at 20 °C. After centrifugation, the bacterial cell pellet was resuspended in 30 ml of buffer A (phosphate-buffered saline, pH 7.2, 1 mM dithiothreitol) containing 4 mM Pefabloc. The cells were broken by French Press (1,500 p.s.i., twice) and centrifuged (60 min, 34,500 × g, at 4 °C), and the resulting supernatant was applied to a glutathione-Sepharose column (Amersham Biosciences). The column was washed with 50 ml of buffer B (20 mM Tris, pH 7.4, 200 mM NaCl). Bound proteins were eluted with buffer C (200 mM Tris, pH 7.4, 200 mM NaCl, 1 mM maltose) containing 10 mg/ml, and stored at −20 °C. The protein was at least 95% pure when analyzed by SDS-PAGE.

Recombinant *Saccharomyces cerevisiae* porphobilinogen synthase (HemB) was produced and purified as fusion protein with the maltose binding protein. *E. coli* BL21(DE3)pLys containing the expression plasmid pMalhemB was grown at 37 °C in LB medium. When cells reached an A600 of 0.6 isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 500 μM. After overnight cultivation at 20 °C the cells were centrifuged, and the cell pellet was resuspended in buffer A (20 mM Tris, pH 7.4, 200 mM NaCl). Cells were broken by sonication, and the soluble protein fraction was loaded onto a 5-ml amylose resin column (Amersham Biosciences). Bound proteins were eluted from the column using a linear NaCl gradient (0–1 M NaCl in 20 mM HEPES, pH 7.2). HemA-containing fractions were pooled, concentrated to 10 mg/ml, and stored at −20 °C. The protein was at least 95% pure when analyzed by SDS-PAGE.

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were stored at 4 °C. Recombinant Bacillus megaterium porphobilinogen deaminase (HemC), recombinant B. megaterium uroporphyrinogen III synthase (HemD), and recombinant Homo sapiens uroporphyrinogen III decarboxylase (HemE) were purified according to previously published protocols (23, 24). For each of these proteins only the first purification step using nickel-nitrilotriacetic acid columns was performed. After elution of the proteins from the nickel-nitrilotriacetic acid column protein-containing fractions were pooled, and the buffer was exchanged against de-aerated buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) using PD10 columns (Amersham Biosciences) in an anaerobic chamber. The concentration of the protein solution was 2 mg/ml. The protein was judged to be at least 80% pure by SDS-PAGE. The protein was stored at 4 °C.

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

**Iron Determination**—The iron content of recombinant, purified E. coli HemN was determined as previously described (6). Iron standards were purchased from Merck.

Synthesis of Coproporphyrinogen III—Coproporphyrinogen III (1) was synthesized by reduction of coproporphyrin III using sodium amalgam (25). Modifications of this procedure were described recently (6).

Synthesis of Deuterium-labeled Coproporphyrins III—[5,10, 15,20,21,22,23,24-2H32]coproporphyrinogen III (2) was synthesized by reduction of coproporphyrin III using sodium amalgam in 50 mM KOD in D2O. MS, [M + H]+: 683.3 Da, determined distribution: M-full: 18.2%, M-full-1D: 21.0%, M-full-2D: 20.6%, M-full-3D: 18.9%, M-full-4D: 13.5%, M-full-5D: 5.8%, M-full-6D: 2%. [2,2,3,3-2H4]ALA, 1 mM [3,3,5,5-2H4]ALA, 150 mM Tris-HCl (pH 8.0) and 150 mM NaCl. The reaction was performed as described for the synthesis of 3, and the freeze-dried products were stored at −20 °C. MS, [M + H]+: 683.3 Da, determined distribution: M-full: 98.6%, M-full-15N: 1.4%.

**Extraction of Coproporphyrinogen III**—The enzymatically synthesized coproporphyrinogen III was isolated in its oxidized form (coproporphyrin III) from the reaction mixtures by solvent extraction. The extraction was performed as described before (27) with some minor modifications. The freeze-dried reaction products were dissolved in 4 ml of H2O, and 20 ml of ethyl acetate/glacial acetic acid (3:1, v/v) was added. This mixture was allowed to stand overnight at −20 °C for protein precipitation. Precipitated proteins were removed by centrifugation. The resulting pellet was washed with 5 ml of ethyl acetate/glacial acetic acid and centrifuged, and the supernatant was added to the main ethyl acetate fraction. The combined fractions were washed twice with 15 ml of saturated sodium acetate solution. The washings were extracted with fresh ethyl acetate (2 ml, 10×), which was added to the main ethyl acetate fraction, and the combined ethyl acetate fractions were washed once with 3 ml of (v/v) sodium acetate. Coproporphyrin III was extracted from the ethyl acetate with 0.36% (v/v) HCl. The concentration of the coproporphyrin III solution was determined by fluorescence spectroscopy using a PE LS50B luminescence spectrometer (PerkinElmer Life Sciences) with an excitation wavelength of 409 nm, emission wavelength scan from 530 to 680 nm, and commercial coproporphyrin III as standard. The coproporphyrin III solution was freeze-dried and stored at −20 °C.

**High Performance Liquid Chromatography Analysis of Porphyrins**—HPLC analysis of porphyrins was performed as described previously (28). Coproporphyrin III, protoporphyrin IX, and uroporphyrin I were used as porphyrin standards.

**Mass Spectrometry of Coproporphyrin III**—Isotope-labeled and non-labeled coproporphyrins were dried down, redissolved in 2% (v/v) methanol, 0.5% (v/v) formic acid, and desalted using ZipTip C18 microcolumns (Millipore GmbH, Eschborn, Germany), following the manufacturer’s instructions. Samples were analyzed in a Waters Micromass matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker, Bremen, Germany) using α-cyano-4-hydroxyacinnamic acid as matrix. The efficiency of isotope labeling was monitored by analyzing the isotope distribution of the coproporphyrins. Effects of the 13C-isotope distribution were subtracted by using the peak-areas of ions of the fully labeled (M-full) and less deuterated (M-half-xD) species.

**Oxygen-independent Coproporphyrinogen III Oxidase Activity Assay**—The activity assay for HemN was performed as previously described (6).

**Preparation of EPR Samples**—Sample preparation was carried out in an anaerobic chamber (5% H2/95% N2, <1 ppm O2). Purified HemN (10 mg/ml) was mixed with sodium dithionite and SAM. The mixture was then transferred to a quartz EPR tube, and the substrate coproporphyrinogen III was added. After mixing the sample was frozen within 90 s in liquid nitrogen. The final concentrations were: 100 μM HemN, 2 mM dithionite, 1.5 mM SAM, and 120 μM coproporphyrinogen III in a final...
volume of 250 μl. For the sample in D₂O the buffer of the HemN solution was exchanged against 50 mM Tris, 3 mM dithiothreitol, 0.1% (v/v) Triton X-100, and 300 mM NaCl in D₂O. The protein solution was reduced by 2 mM dithionite and 1.5 mM SAM. To this mixture a solution of coproporphyrinogen III in 50 mM Tris and 3 mM dithiothreitol in D₂O was added. The sample was transferred to an EPR tube and frozen in liquid nitrogen.

**EPR Measurements**—EPR spectra were recorded with an EMX-6/1 X-band spectrometer (Bruker, Karlsruhe, Germany) with a standard TE102 rectangular cavity and an ESR-900 helium flow cryostat with variable temperature (Oxford Instruments, Oxford, UK). From a spectrum of a strong pitch standard (g = 2.0028) and the microwave frequency as detected by the built-in ER-041–1161 microwave frequency counter, an actual magnetic field at the sample 0.35 ± 0.05 mT higher than that measured by the EMX-032T Hall Field probe was determined. The magnetic field of the recorded spectra was corrected accordingly. For spin integration a solution of 10 mM CuSO₄ in 2 M NaClO₄ and 10 mM HCl was used. Note that it was not possible to improve the spectral resolution by mathematical treatment (22).

**RESULTS AND DISCUSSION**

**EPR Spectrum of a Potential Substrate Radical for Oxygen-independent Coproporphyrinogen III Oxidase HemN**—Recently, it was shown that SAM cleavage into methionine and a putative 5'-deoxyadenosyl radical was dependent upon electron transfer from the [4Fe-4S] cluster of HemN (17). The next stage in the catalytic process was proposed to be the stereospecific hydrogen abstraction from the propionate side chain of ring A of the substrate coproporphyrinogen III (6). To investigate whether organic radicals are intermediates during the HemN reaction, we performed various EPR measurements of reduced [4Fe-4S] cluster containing enzyme samples in the presence of SAM and coproporphyrinogen III. An EPR signal characteristic for an organic radical (gav = 2.0029 ± 0.0002) was observed at 145 K for a sample that contained dithionite-reduced HemN, SAM, and coproporphyrinogen III (Fig. 1, trace C). Various control experiments were performed, and the signal did not appear for samples of dithionite-reduced HemN alone (Fig. 1, trace A), reduced HemN with added SAM (Fig. 1, trace B), or samples containing the complete mixture in which HemN was absent (data not shown). Although slightly saturated, the organic radical signal was also detected at 5 K with the lowest microwave power attainable (2 micro-watts). No obvious sharpening or broadening was observed compared with the spectrum recorded under non-saturating conditions at temperatures up to 160 K (data not shown). Dithionite-reduced HemN (100 μM), in the absence of coproporphyrinogen III, exhibited a very weak EPR signal at 5–30 K with g = 2.06 and 1.93. This signal (~0.01 spin/HemN) disappeared upon addition of coproporphyrinogen III and SAM. No S = 3/2 EPR signal was seen under conditions appropriate for detection (4–20 K, 50-milliwatt microwave power, and 1.25-mT modulation). Similar observations have been made with lysine-2,3-aminomutase where the oxidation of the [4Fe-4S]⁺⁺ cluster is concomitant with the generation of lysyl (22) or anhydrodienydosyl radicals (29). The relaxation of the organic radical in HemN (Pav = 5 milliwatts at 150 K and detectability at 5 K) indicated that despite the absence of a reduced cubane a slightly enhanced relaxation occurred. At low temperatures the [4Fe-4S]⁺⁺ cluster is diamagnetic but, depending on the strength of the J-coupling, the thermally excited spin states (S = 1, S = 2, etc.) can become populated. As postulated for lysine-2,3-aminomutase these paramagnetic excited states lead to enhanced dipolar relaxation (30). In addition to the J-coupling the distance of the radical to the oxidized cubane plays an important role. It can be estimated from coproporphyrin III modeled into the HemN structure (7) that the distance of the A or B ring to the non-cysteinyl-coordinated iron ion of the [4Fe-4S]⁺⁺ cluster is 12–16 Å. Apparently such a distance leads to enhanced relaxation but not to blurring of the spectrum upon raising the temperature. The same holds true for lysyl and analogous radicals in lysine-2,3-aminomutase, which were recorded at 77 K and a relatively high microwave power (4 milliwatts (22)). The blurring of the signal of the allylic anhydroadenosyl radical in lysine-2,3-aminomutase above 30 K seems to deviate from the latter. However, this species is anticipated to occupy a position “halfway” between the cubane and the lysyl radical (i.e. 6 – 8 Å to the cubane) and thus experiences the excited states at a much closer distance.

During our experiments we observed maximal signal intensity for the detected radical species when the samples were frozen within 90 s after mixing of reduced HemN, SAM, and coproporphyrinogen III. Incubation of 60 s or 180 s led to slight losses of integrated signal intensity (16 and 24% loss, respectively). Depending on the HemN preparation, the observed integration of the radical signal varied from 0.025 to 0.11 spin per HemN. Changes in the incubation time and the ratio of coproporphyrinogen III to HemN did not systematically increase the intensity. Two explanations for the low integrated intensity (0.025–0.11 spin per HemN) can be put forward: First, the substrate radical is inadvertently lost by diffusion out of the protein. Subsequently the SAM-dependent regeneration of a new radical takes place at a steady-state level leading to the observed stoichiometry. Second, the observed substratochiometry may relate to the extent of ditionite reduction of the [4Fe-4S] cluster. Both mechanisms could also be operative at the same time. Because reduction of the [4Fe-4S] cluster of HemN by dithionite in the presence or absence of SAM proved difficult (see above) at least mechanism 2 seems to be operative. In the study of the lysyl-radical generated by lysine-2,3-aminomutase higher stoichiometries (0.1–0.55 substrate radical/subunit) were reported (31). The latter system, however, differs from HemN, because generation of the 5'-deoxyadenosyl radical by SAM is followed by multiple turnovers contrary to HemN, which requires one SAM per decarboxylation (17).

In all experiments performed with non-isotopically labeled coproporphyrinogen III in H₂O the spectral shape of the radical signal (i.e. the complex pattern of resolved hyperfine splittings) remained constant.
Thus, there is no compelling evidence for the presence of multiple species. Several reasons might be responsible for the observed stability of the substrate radical. If hydrogen abstraction occurs on the \( \beta \)-carbon of the propionate side chain, the resulting substrate radical is likely to be of an allylic nature. Therefore, the stabilization would be mediated by the delocalization of the unpaired electron with the mesomerization of the \( \beta \)-C atom and the olefinic bond of the pyrrole ring (C5–C6 or C8–C9). Moreover, the final electron acceptor, required for product formation, is absent from the EPR sample mixture, which further traps the radical on the substrate. The earlier observed stereospecific removal of the \( \beta \)-pro-S hydrogen by the anaerobic coproporphyrinogen III oxidase of \( R. \) sphaeroides (18) is compatible with the radical mechanism outlined above (Scheme 1c). Stereospecific abstraction of a hydrogen atom from the methylene moieties by an adenosyl radical has been extensively documented for adenosyl cobalamin-dependent enzymes like diol dehydratases and carbon-skeleton-rearranging mutases (32). However, this property is not confined to adenosyl radicals generated from adenosylcobalamin, because (pro)chiral substrates and products are also handled by the adenosyl radical generated by the [4Fe-4S]\(^{15}\) cluster/SAM combination in Radical SAM enzymes.

Enzymatic Synthesis of Deuterium- and \(^{15}\)N-Labeled Coproporphyrinogen III—To investigate further the observed potential substrate radical we synthesized various deuterium- and \(^{15}\)N-labeled coproporphyrinogens. For this purpose a complete enzymatic synthesis of coproporphyrinogen III from differentially labeled precursors (ALA and succinyl-CoA) was performed (Scheme 2). Using this procedure the non-labeled \( 1 \) and the labeled coproporphyrinogens \( 2 \–6 \) were synthesized (Fig. 2). Although the use of purified multienzyme systems has been employed to regiospecifically label intermediates in vitamin B\(_{12}\) biosynthesis, this is the first time that such an approach has been used in the synthesis of intermediates of porphyrin biosynthesis.

For the synthesis of \( 1 \), \( 3 \), and \( 6 \) ALA, \([3,3,5,5-\text{D}_4]\)ALA, and \([^{15}\text{N}]\)ALA, respectively, were employed as starting material. The reactions included four coupled enzymatic steps catalyzed by the enzymes HemB, HemC, HemD, and HemE. For the synthesis of \( 3 \) one additional chemical step was performed, the enolization of ALA in D\(_2\)O. After the 48-h incubation of ALA in D\(_2\)O (see “Materials and Methods”) the solution was pale yellow, which indicated that ALA had also autocondensed to pyrazine and pseudoporphobilinogen (33) during the reaction. \(^1\)H NMR spectroscopy showed that the remaining ALA had undergone complete hydrogen exchange at C5 and C6 to give \([3,3,5,5-\text{D}_4]\)ALA (data not shown). This material was used for the enzymatic synthesis of \( 3 \). For the synthesis of \( 4 \), \([2,2,3,3-\text{D}_4]\)succinyl-CoA was chemically synthesized via the reaction of \([\text{H}_4]\)succinic anhydride with coenzyme A. The resulting \([2,2,3,3-\text{D}_4]\)succinyl-CoA, together with glycine, were used as substrates in an incubation that included five coupled enzymatic steps catalyzed by HemA, HemB, HemC, HemD, and HemE. After the enzymatic reaction mixtures were left at 37 °C overnight (see “Materials and Methods”) the solutions were found to vary in color from pale red (3) to intensive pink (1 and 4) indicating that some oxidation of the porphyrinogens had taken place. Subsequent HPLC analysis of the enzymatic reaction mixtures showed that the main porphyrin produced in all the mixtures was coproporphyrin III with a retention time of 6.3 min (Fig. 3, A–D). A minor peak in the HPLC chromatogram at a shorter retention time of 5.7 min was observed representing residual...
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FIGURE 3. HPLC analyses of porphyrins produced during the enzymatic syntheses of coproporphyrinogen III. A–D, porphyrins produced during the syntheses of substrates 1 (A), 4 (B), 6 (C), and 3 (D), respectively. E, HPLC analysis of porphyrins after solvent extraction (see “Materials and Methods” and elsewhere in the text for details). Coproporphyrin III (retention time = 6.3 min) and uroporphyrin I (retention time = 5.4 min) were used as porphyrin standards.

uroporphyrin III intermediate. To separate the coproporphyrin III from the uroporphyrin III and the enzymes a solvent extraction of coproporphyrin III was performed. After this solvent extraction coproporphyrin III was the only porphyrin observed by HPLC analysis (Fig. 3E). The amount of coproporphyrin III recovered after the solvent extraction was determined by fluorescence spectroscopy. The synthesis of 1 resulted in 520 nmol of coproporphyrin III (42% yield with respect to ALA). The syntheses of 3 and 6 yielded 200 nmol (16% yield) and 366 nmol (29% yield), respectively, of labeled coproporphyrin III. For the synthesis of 4, in which labeled succinic anhydride was used as starting material, 500 nmol of labeled coproporphyrin III were obtained (17% yield with respect to succinic anhydride). For EPR analysis coproporphyrinogens 1, 3, 4, and 6 were reduced to the corresponding coproporphyrinogens by sodium amalgam treatment. For the syntheses of 2 and 5 coproporphyrins 1 and 4 were reduced by sodium amalgam in 50 mM KOD/D_2O, which resulted in the incorporation of deuterium both at the methylene bridge positions (C^5, C^{10}, C^{15}, and C^{20}) as well as at the pyrrole nitrogen atoms of the macrocycle. The crystal structure of uroporphyrin III decarboxylase in complex with its product revealed that coproporphyrin III is a basket-shaped asymmetric molecule in which the hydrogen atoms of the methylene bridges are chemically inequivalent (see coproporphyrinogen III in uroporphyrinogen decarboxylase, PDB 1R3Y (34)). Thus, applying the principle of microscopic reversibility, reduction of the relatively planar coproporphyrin III could still lead to incorporation of deuterium mainly into one of the two possible positions in the methylene bridges. This issue was not further pursued experimentally, but it is relevant for the interpretation of the following EPR results.

The incorporation of deuterium into the synthesized substrates was analyzed by mass spectrometry of the corresponding coproporphyrinogens after the solvent-extraction step (1, 3, 4, and 6) or amalgam reduction in KOD/D_2O (2 and 5). On the basis of the mass spectra, deuterium and ^15N incorporation into the various coproporphyrinogen III molecules was judged to be successful for all synthesized substrates (for detailed results consult “Materials and Methods”). However, a low rate of D/H exchange was observed for all deuterium-labeled coproporphyrins, which most likely occurred during the enzymatic reaction and the subsequent solvent extraction, because these procedures were carried out in non-deuterated buffers and solvents.

Deuterium and [^15N]Coproporphyrinogen III Molecules Are Efficient HemN Substrates—All of the synthesized coproporphyrinogen III molecules were tested for their ability to serve as substrates in the HemN reaction. Product formation was followed by fluorescence spectroscopy and HPLC-based product analysis as described in detail before (6). All labeled substrates were completely transformed into the reaction product protoporphyrinogen IX by HemN. Formed products were all efficient substrates for purified recombinant Thermosynechococcus elongatus protoporphyrinogen IX oxidase, the next enzyme in the tetrapyrrole biosynthetic pathway, tested as described before (17). Mass spectrometric analysis of the enzymatically oxidized HemN reaction product of compound 4 revealed the expected molecular masses. These results demonstrate that the synthesized compounds efficiently participated in the HemN-catalyzed reaction and yielded the expected products.

EPR Spectra of the Labeled Substrates—The complex hyperfine splitting pattern of the observed radical signal (Fig. 1C) indicated the coupling of the unpaired electron with several hydrogen atoms. To investigate these interactions EPR spectroscopy with the different labeled substrate molecules was performed. Because the protons of certain organic radicals are subject to exchange with solvent (i.e., the α-H in the glycyl radical of pyruvate formate-lyase (35) and various organic radicals (36)), it is appropriate to show that selective incorporation of deuterium is not abolished by washout. In Fig. 4 the spectra of the non-labeled substrate in H2O and D2O (traces A and B, respectively) are compared. Without artificially trading down the signal-to-noise ratio, spectra were recorded with a field modulation of 0.4 mT. Lower modulation amplitudes did not lead to increased resolution (data not shown). Obviously, none of the strongly coupled hydrogen atoms of the radical was subject to solvent exchange. However, the spectrum in D2O shows an increased overall resolution as indicated by the presence of more pronounced
shoulders in the central part of the EPR envelope (336.6 and 338.2 mT, Fig. 4, trace B) and increased almost zero-crossing "depths" at 335.6 and 339.2 mT (Fig. 4, trace B). This increased resolution could be caused either by a general reduction of the inhomogeneous broadening by solvent exchangeable protons in the vicinity of the radical or by a specific but weak coupling with one or more pyrrolic N–H hydrogen atoms. The latter was tested by substitution of the pyrrolic nitrogen atoms in coproporphyrinogen III by 15N (substrate 6, Fig. 4, trace C). Within experimental error, the spectrum was almost identical to that of non-labeled coproporphyrinogen III. Thus the spin density at the pyrrolic nitrogen is very low. This observation implies that the D2O-induced sharpening is caused by the general reduction of the inhomogeneous broadening.

The spectra of the deuterium-labeled substrates were compared with that of non-labeled coproporphyrinogen III (Fig. 5). The most dramatic effect of labeling on an EPR spectrum was observed for a substrate in which almost all hydrogen atoms of coproporphyrinogen III (including pyrrolic N–H) had been replaced by deuterium atoms (substrate 5). For this substrate the hyperfine splitting of the radical signal collapsed almost completely (Fig. 5E). Because substrate 5 still had one 1H-methylene bridge proton originating from the coproporphyrin III methine group before D2O/sodium amalgam reduction this proton apparently did not couple very strongly ($A_{\text{av}} < 0.6$ mT). As already mentioned the cup-shaped nature of coproporphyrinogen III renders the methylene protons chemically inequivalent. The application of the principle of microscopic reversibility would thus implicate that sodium amalgam reduction in D2O stereoselectively introduced a deuterium atom at the methylene bridge position. The above hypothesis was tested by the use of substrate 4 for EPR (Fig. 5D). The introduction of a hydrogen atom into the methylene bridge by H2O/sodium amalgam reduction of coproporphyrin 4 (yielding substrate 4) led to a single well defined hyperfine splitting ($A_{\text{av}} = 1.2$ mT). Because the pyrrole N–H was shown not to contribute to coupling of this order of magnitude, the organic radical in HemN revealed substantially different hyperfine couplings for the two methylene bridge protons. Such angular/dihedral dependence is typical for protons of β-methylene groups in π-type radicals (37, 38) and assigns a significant spin density to the carbon atom between the methylene bridge and the pyrrole nitrogen (C4 or C5). The same effect of deuterium introduction into the methylene group of substrate 1 by sodium amalgam/D2O reduction was observed for substrate 2 (Fig. 5B). In this case the differences between 2 and the non-labeled substrate were also only found for the D/H atoms at the methylene bridge positions and the pyrrole nitrogens. The spectrum of 2 (Fig. 5E) appeared as a triplet ($A_{\text{av}} = 2.3$ mT). In addition to the 1.2 mT coupling of a specific methylene bridge proton two relatively strong 1H-couplings were observed. Finally, comparison of the spectrum of 4 (Fig. 5D) with the spectrum of 3 (Fig. 5C), for this substrate the hydrogens at the α-positions of the propionate side chains were not exchanged, indicated that there was also coupling with the α-protons of the propionate side chains. This conclusion depends on the observation described above that one of the methylene bridge protons did not couple strongly (see spectrum of 5, Fig. 5E).

Therefore, the spectrum of substrate 3 also indicated the presence of spin density at the β-carbon of the propionate side chain, which is in accordance with the proposed hydrogen atom abstraction from this position.

The observed changes in the EPR spectra of the deuterium-labeled coproporphyrinogen III molecules clearly identify the radical signal as substrate derived. Additionally, they show that the substrate radical is an allylic radical with spin density on the β-carbon of the side chain as well as on the ring C atom between methylene bridge and pyrrole nitrogen (Scheme 3).

**CONCLUSIONS**

The results of this investigation in combination with previous findings concerning the catalytic properties of HemN allow a refined detailed view on its enzymatic mechanism. During the initial step the [4Fe–4S] cluster of HemN is reduced by a cellular reduction system. The reduced iron-sulfur cluster transfers its electron to SAM, which is thereby homolytically cleaved to methionine and the putative 5′-deoxyadenosyl radical. Mössbauer spectroscopy and the solved HemN crystal structure identified the direct interaction of one of the two SAM molecules with the iron-sulfur cluster. This structural arrangement seems to be necessary for the fast and safe transfer of the electron to SAM. Economically, this electron transfer occurs in vivo only in the presence of the substrate (17). Subsequent hydrogen abstraction occurs at the β-carbon of the substrate propionate side chain and results in the formation of a substrate radical, as demonstrated by the results we pres-
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The observed allylic nature of the resulting substrate radical is in good agreement with the proposed mechanism of hydrogen abstraction at the β-carbon of the propionate side chain. In our EPR experiments we exclusively observed substrate radical derived signals. No indications for a protein derived radical species were obtained. Therefore, the 5′-deoxyadenosyl radical most likely directly abstracts the hydrogen atom from the propionate side chain of the substrate. Once the substrate radical is formed the first of two decarboxylation reactions can take place. For this step an electron acceptor is required (Scheme 1c). Although the substrate radical exhibited quite a long lifetime in our EPR experiments, this is very unlikely for the in vivo situation. In vivo this electron acceptor is probably bound in or close to the active site of the enzyme, which allows for instant reaction with the substrate radical. An appropriately located electron acceptor binding site was indeed deduced from the solved crystal structure. However, one has to keep in mind that two SAM molecules are cleaved during one catalytic turnover and two SAM molecules were found in the active site of the HemN crystal structure. Mutagenesis of the SAM2 binding site revealed the essential function of this second SAM molecule for catalysis (17). In a first model for the following steps one can propose two independent SAM cleavage events utilizing two electrons both coming from the iron-sulfur cluster. However, for this proposal the question of the path of the electron to SAM2 remains to be solved. Because the crystal structure represents an open conformation of the enzyme with no substrate bound, additional amino acid residues might enter the active site upon substrate binding and active site closure and might mediate the necessary electron transfer. Alternatively, SAM2 might serve directly as the electron acceptor during the first decarboxylation reaction. By using SAM2 as electron acceptor not only the first decarboxylation step would be executed but also the second reductive cleavage of a second SAM molecule with concomitant 5′-deoxyadenosyl radical formation would be accomplished. This second deoxyadenosyl radical could then abstract a hydrogen atom from the second substrate propionate side chain again leading to formation of a substrate radical. Finally, the second decarboxylation can only take place in the presence of an electron acceptor, which in this case is different from SAM2 and remains to be identified.

Here we provide the second example for the functional role of a substrate radical during Radical SAM enzyme catalysis. Both investigated model enzymes, LAM and HemN, are highly diverse in their catalyzed reactions, utilized substrates, and employed cofactors. Nevertheless, the described findings now allow the proposal of the general importance of substrate radical formation for Radical SAM enzyme catalysis.

Acknowledgments—We thank Profs. Wolfgang Buckel (Institut für Mikrobiologie, Philipps Universität Marburg, Germany) and Bernard T. Goldberg (School of Natural Sciences, University of Newcastle upon Tyne, UK) for helpful discussions. We are grateful to Prof. Rolf K. Thauer for the use of the EPR spectrometer (Max-Planck-Institut für Terrestrische Mikrobiologie, Marburg, Germany).

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