BIOORGANIC CHEMISTRY 22, 284–293 (1994)

Iron Uroporphyrin I and a Heme c-Derivative Are Prosthetic Groups in *Desulfovibrio gigas* Rubredoxin Oxidase[1]

RUSSELL TIMKOVICH,*[,2] ROBERT S. BURKHALTER,* ANTONIO V. XAVIER,† LIANG CHEN,‡ AND JEAN LEGALL‡

*Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487-0336; †Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Oeiras, Portugal; and the ‡Department of Biochemistry, University of Georgia, Athens, Georgia 30602

Received April 11, 1994

Rubredoxin-oxygen oxidoreductase (ROO) from the sulfate-reducing bacterium *Desulfovibrio gigas*, is an unusual terminal oxidase capable of reducing molecular O_2 to water. The nature of its heme prosthetic groups has been investigated. It was found to contain two distinct hemes. One is Fe uroporphyrin I. The second is closely related to heme c. It is an Fe protoporphyrin IX where two electrophilic groups have added covalently across the double-bonds of the former 3,8 vinyl groups (IUB-IUPAC Tetrapyrrole numbering). These covalent attachment bonds are not to the main polypeptide chain, but instead are to an unidentified component. This component is soluble in acidic acetone, but cannot be extracted into organic solvents that typically solubilize free heme groups. Preliminary evidence suggests that the covalent bonds are not thioether bonds, but they are acid labile.

Desulfovibrio are sulfate-reducing bacteria that have traditionally been considered to be strict anaerobes. Therefore it was surprising when it was discovered that they could utilize polyglucose for the formation of ATP linked to the reduction of O_2 to water (*1*). A novel flavin-containing hemoprotein, rubredoxin-oxygen oxidoreductase (ROO), was found to participate in an electron transport chain linking NADH oxidation to O_2 reduction (*2*). It is reduced by the iron-sulfur protein rubredoxin and reoxidized by O_2. The reaction which leads to the formation of water is not inhibited by cyanide or azide, but is affected by CO. ROO is a soluble homodimer (43 kDa/monomer) that was shown to contain FAD as well as heme. The iron analysis indicated 1.4 ± 0.3 g-atoms Fe per monomer; therefore, there were multiple hemes per enzyme, but the precise composition was ambiguous. The heme or hemes were not identified, but the uv-visible spectra suggested that there might be novel aspects to the structure and/or coordination sphere.

The nature of the heme prosthetic groups has now been investigated in more depth, subject to limitations imposed by the scarcity of the enzyme. ROO is present only at the level of circa 40 mg/kg cells, and the cells grow to a density

[1] Supported in part by National Institutes of Health Grant GM 43292 (RT) and European Economic Community Grant P.L. 932052 (AVX and JL).

[2] To whom correspondence should be addressed.

0045-2068/94 $6.00

of about 1 g wet wt/liter. Within this limitation, substantial progress has been made and this report establishes that two distinct hemes are associated with ROO.

EXPERIMENTAL

ROO was purified as described previously (*2*). Only 20 mg were available for heme extraction and identification studies. This had to include various test procedures of variable success as well as the final purification of material for spectroscopic analysis. Acid acetone extraction conditions, iron removal reactions, and esterification procedures have been described (*3*). Conditions for HPLC separations, NMR, and liquid secondary ion mass spectrometry (LSIMS, also known as fast atom bombardment, or FAB) have also been reported (*4*). During the course of this work, it became evident that standards for comparisons would be required. Uroporphyrin I and III as the dihydrochloride salts were purchased from Porphyrin Products, Logan, Utah. They were further purified by esterification and HPLC and then converted back to carboxylic acids by room temperature incubation for 24 h under nitrogen in 6 M HCl, followed by vacuum drying. Iron was inserted into uroporphyrin by a standard ferrous sulfate method (*5*), with some additional modifications for purification. The crude Fe-uroporphyrin was prepared in 1- to 2-μmol amounts and added to ethylacetate and the solution was extracted once with an equal volume of 1 M HCl. The organic layer was dried under vacuum. The residue was dissolved in a minimal amount of 1 : 1 pyridine : water and applied to a 0.7 × 3.5-cm column of DEAE cellulose that had been equilibrated versus 10 mM Tris–hydrochloride, pH 8. The column was washed with 50 mM potassium phosphate, pH 7, and then an aqueous solution of acetic acid (5% v/v). Fe-uroporphyrin was then eluted with 20% acetic acid and dried under vacuum.

Porphyrin c (scheme as in **3** with R = −OH groups) was prepared as described previously by adaptation of Paul's silver sulfate method (*6*). The OCH_3 derivative was prepared by the following procedure. The porphyrin c methyl ester was dissolved in tetrahydrofuran (1 μmol/10 ml) and continuously purged with dry nitrogen. Hexane-washed NaH (100 mg, Aldrich Chemical Co.) was added, followed by 0.5 ml of methyl iodide. Hydrogen gas evolution occurred, mostly due to residual water in the solvents. After 5 min, the reaction mixture was poured into equal volumes of benzene and water and stirred vigorously to quench the excess NaH. The benzene layer was separated, washed with water, and dried. The derivative was purified by HPLC of the dried residue on silica gel eluted with 3 : 7 hexane : chloroform, where it chromatographed as a well-resolved peak with capacity factor of 2.3.

RESULTS

The ferrous alkaline pyridine hemochromogen of ROO gives characteristic α, β, and Soret bands as shown in Fig. 1A. However, there are complicating features due to the flavin, and an unidentified feature at 585 nm. The α band maximum

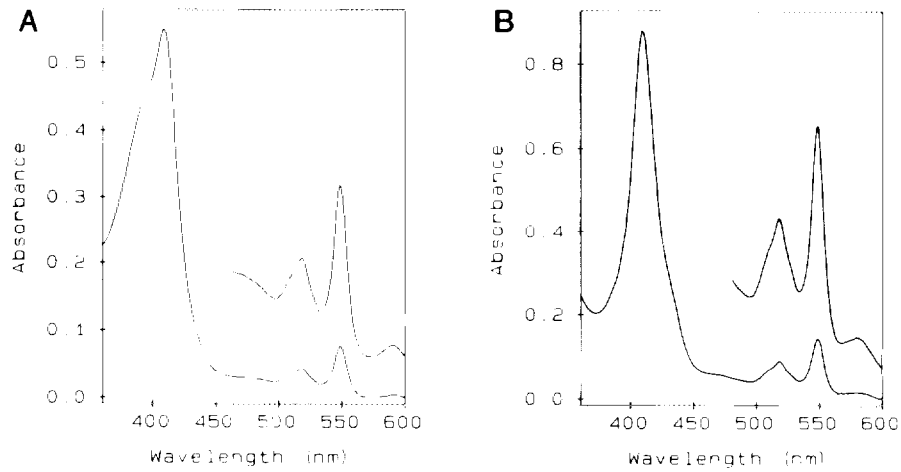


FIG. 1. (A) The ferrous alkaline pyridine hemochromogen visible spectrum of ROO. (B) Synthetic Fe uroporphyrin I under the same conditions.

was measured in spectra taken with narrow slits (0.25 nm), recorded in the first- and second-derivative mode, and determined to be 548.6 ± 0.3 nm. The pyridine hemochromogen of synthetic Fe-uroporphyrin, shown in Fig. 1B, matches the main features of ROO very well including an α maximum at 548.8 ± 0.3 nm. Unfortunately, this is not sufficient to prove identity. Other iron porphyrins where the substituents do not contain vinyl or formyl groups or other strongly electronegative or electrophilic groups would also be expected to have a maxima around 550 nm, as in the well-known case of heme c. Weak bands are observed in the synthetic compound in Fig. 1B, especially around 590 nm. These are not part of the electronic spectrum of Fe-uroporphyrin, but are degradation products or mixed ligand species. This suggests that the feature in ROO at 585 nm could be an artifactual by-product, but the intensity observed in ROO is much higher than we have observed in any synthetic sample, and so the 585 band may still represent a distinct chromophore of unknown structure.

Acid acetone extraction of ROO produces a white precipitate and an orange-yellow supernatant. We have been unable to resolubilize the denatured protein for sensitive optical spectroscopy, but visually it seems devoid of pigment. The acidic acetone supernatant has broad band maxima at 632, 585, 535, and 370 nm. The broad Soret with shoulders around 410 nm is attributable to heme mixed with FAD. The 632 and 535 features are typical for high spin ferric hemins and do provide further structure evidence. The reappearance of the 585-nm band in the acidic acetone spectrum argues that it is not a by-product of the alkaline pyridine hemochromogen conditions.

Ethylacetate extraction of the acetone solution produces an orange organic layer and a bright yellow aqueous layer. Figure 2 shows that the ROO product (Fig. 2A) matches the hemin form of Fe-uroporphyrin (Fig. 2B) very closely. The spectrum of the aqueous layer is dominated by intense broad bands around 385

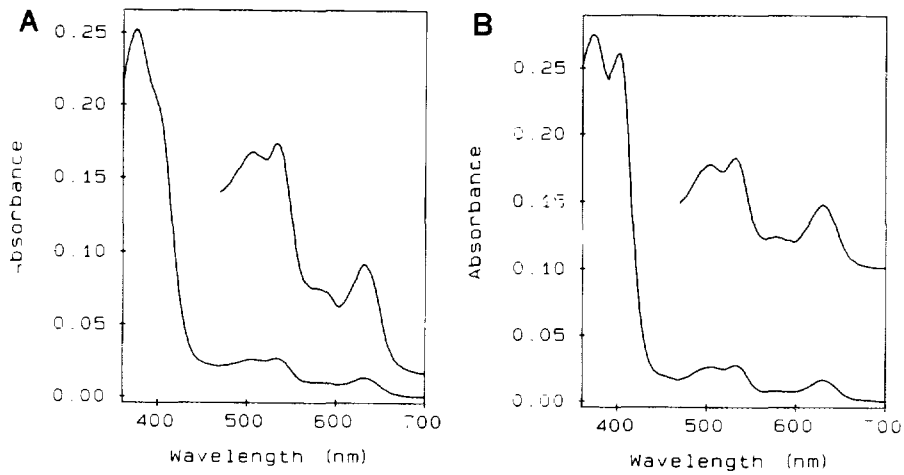


FIG. 2. (A) Visible spectrum of the ethylacetate-soluble heme of ROO in acidic ethylacetate. (B) Synthetic Fe uroporphyrin I under the same conditions.

and 450 nm due to the flavin, and no useful heme characteristics were observed against this intense background.

After iron removal, esterification, and concentration of the ester derivative, HPLC showed that the ROO-derived product matched the retention behavior of uroporphyrin ester as shown in Fig. 3, and possessed a typical uroporphyrin ester visible spectrum. The chromatographic conditions employed would not resolve the

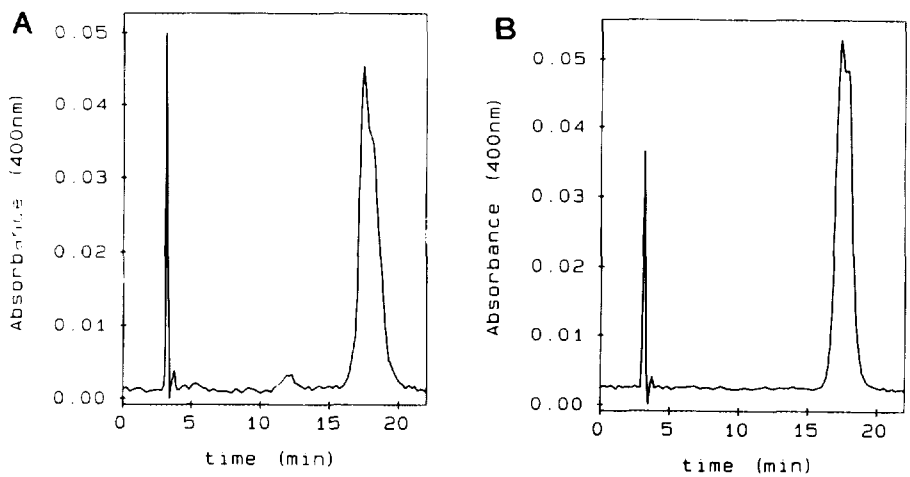
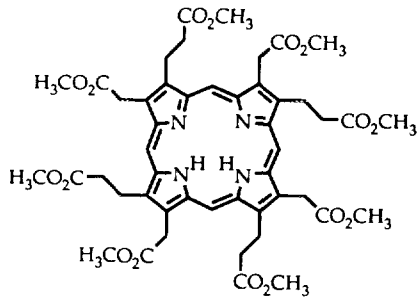


FIG. 3. (A) HPLC chromatogram of the porphyrin methyl ester derived from ROO as the ethylacetate-soluble heme. The column was a standard analytical-size column packed with 5-μm silica gel and eluted with neat chloroform. The small shoulder on the late side of the main peak at circa 18 min is due to an chromatographic injection artifact. (B) HPLC chromatogram of uroporphyrin I methyl ester.



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SCHEME 1

geometrical isomers of uroporphyrin. Low-resolution LSIMS spectra established a molecular ion of m/z of 943 for $(M + H)^+$, as expected for uroporphyrin ester. As the evidence was accumulating implicating Fe-uroporphyrin as a heme in ROO, we had been assuming that it would be the common porphyrin metabolite uroporphyrin III. The NMR spectrum of the ROO product came as a shock, when it clearly showed that the porphyrin was the type I isomer, as shown in Scheme **1** and Fig. 4. The type I isomer is fourfold symmetrical and shows one resonance

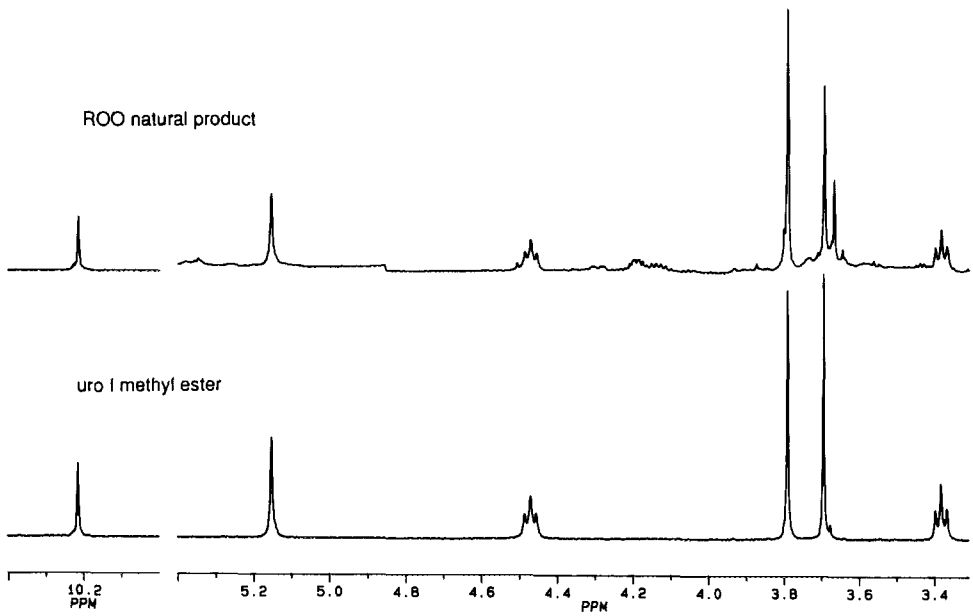
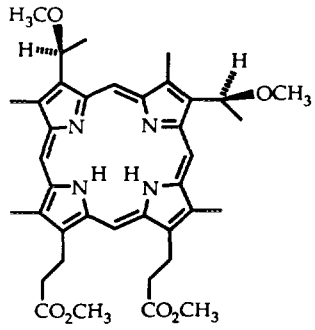


FIG. 4. The 500 MHz ^{1}H-NMR spectra in deuterated chloroform at 295 K. The top panel displays the spectrum of the methyl ester derivative of the ethylacetate-soluble heme from ROO. The bottom panel is commercial synthetic uroporphyrin I methyl ester.



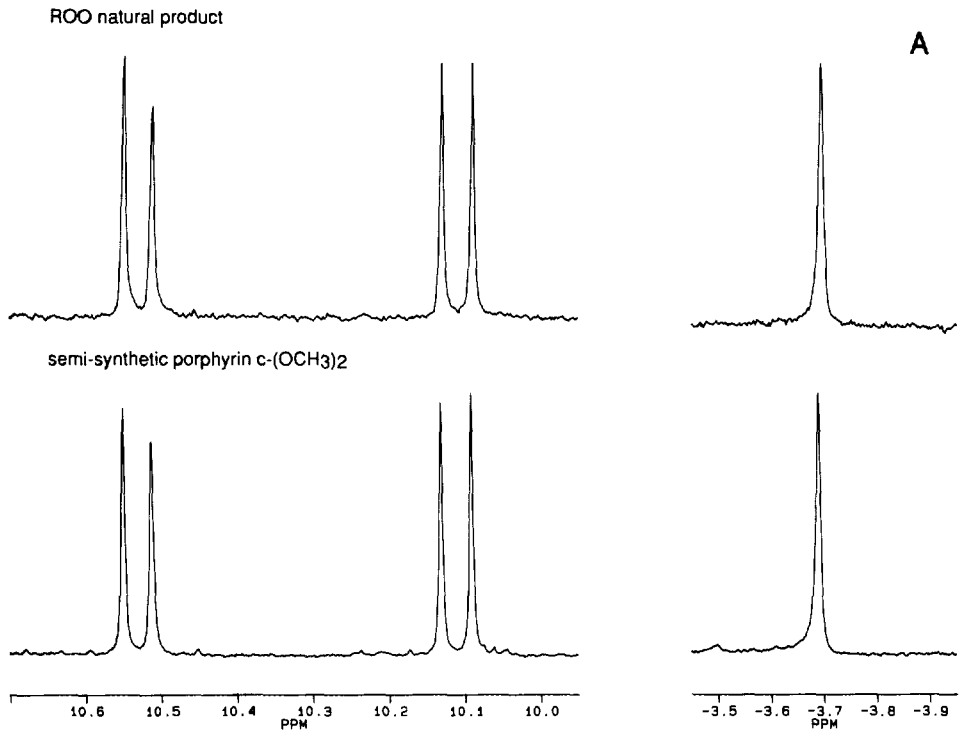
2

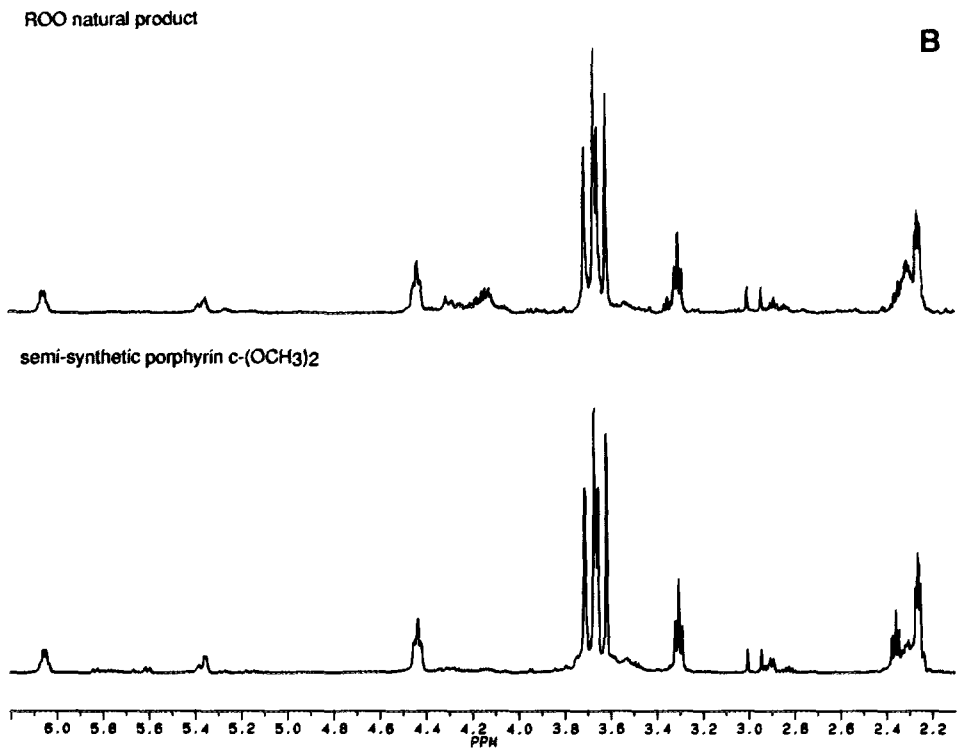
SCHEME 2

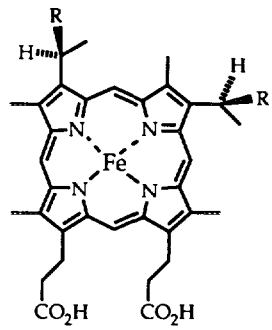
each for the meso, propionate, acetate, and ester groups on the four magnetically equivalent pyrroles. The type III isomer is nonsymmetrical and shows resolved or partially resolved resonances for the inequivalent meso and pyrrole substituents.

A second distinct porphyrin was discovered by the following experiments. The aqueous layer from the ethylacetate extraction of the original acidic acetone extract was lyophilized and then incubated at room temperature for 96 h in 5% (v/v) sulfuric acid in methanol. After the standard workup for porphyrin esters, HPLC on silica gel gave an ester that eluted as a single peak at a capacity factor of 2.33 in 30:70 hexane:chloroform. An additional minor peak was washed from the column in neat chloroform and identified by LSIMS as uroporphyrin ester. Almost certainly this minor amount came from residual Fe-uroporphyrin not extracted in the ethylacetate treatment, but it is important to note that none of the second porphyrin was detected in the main uroporphyrin fraction and only appeared after prolonged acid methanolysis of the water-soluble fraction.

The visible spectrum of the new porphyrin ester showed a typical etio-type pattern with band maxima at 623, 568, 533, 499, and 402 nm. High-resolution LSIMS gave a protonated molecular ion $(M + H)^+$ of observed mass 655.350, while the calculated mass is 655.34956 for the empirical formula $C_{38}H_{47}N_4O_6$. This formula can be thought of as protoporphyrin IX with the addition of two equivalents of CH_3OH, corresponding to the Scheme shown as **2**. Critical features of the NMR of the ester derivative, shown in Fig. 5, were the quartet-doublet spin systems at 6 and 2.2 ppm which were highly similar to –CHRCH3 groups (Scheme **3**) in hematoporphyrin and covalently bound heme c (*6*). There were additional methyl resonances for the new product in the 3.6- to 3.8-ppm range with an intensity corresponding to about six additional protons, which would be consistent with two OCH_3 groups. The complete spectrum was attributed to the Scheme **2** and assigned as follows: 10.53, 10.51, 10.13, and 10.09 ppm, 1H each, s, meso; 6.05 ppm, 2H, q, J = 5 Hz, $-\mathbf{CH}(OCH_3)CH_3$; 2.27 and 2.25 ppm, 3H each, d's, J = 5 Hz, $-CH(OCH_3)\mathbf{CH_3}$; 4.43 and 3.30 ppm, 4H each, t, J = 7.6 Hz, $-\mathbf{CH_2CH_2}COOCH_3$; 3.709 (3H), 3.706 (3H), 3.667 (6H), 3.658 (3H), 3.650 (3H), 3.613 (6H) ppm, overlapping singlets with estimated areas given in parentheses


ROO natural product
A
semi-synthetic porphyrin c-(OCH3)2
10.6 10.5 10.4 10.3 10.2 10.1 10.0
PPM
-3.5 -3.6 -3.7 -3.8 -3.9
PPM


ROO natural product
B
semi-synthetic porphyrin c-(OCH3)2
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2
PPM



3

SCHEME 3

corresponding to the four-ring, two-ester, and two-OCH_3 groups; −3.69 ppm, 2H, br s, NH. To completely confirm the assigned structure, a semi-synthetic sample of **2** was prepared from horse heart cyt c as described under Experimental. As shown in Fig. 5, the match to the ROO product is excellent.

The semi-synthetic sample has two chiral centers and is optically active since the heme removal methods employed do not racemize these centers (*7*). The NMR match to the ROO product strongly suggests that the ROO product is not a diastereomer of heme c at the chiral carbons, but it could not rule out whether the ROO product was the mirror image. Circular dichroism spectra were obtained on the two products. While the signals from the ROO sample were weak due to lack of material, the signs of the ellipticities established that the two were the same isomer.

DISCUSSION

Iron analysis had indicated 1.4 ± 0.3 atoms of Fe per monomer of ROO. The yields of extractable heme and final purified ester derivatives from microscale experiments are not reliable for quantification of heme content, but since ROO is a homodimer, symmetry would suggest two distinct hemes per monomer. Finding two porphyrins in purified ROO is consistent with the observation of two discrete sets of EPR signals in the ferric enzyme (M. Teixeira and A. V. Xavier, personal communication), although the EPR data could conceivably arise from the same

FIG. 5. The 500 MHz ^{1}H-NMR spectra in deuterated chloroform at 295 K. The top panel displays the spectrum of the methyl ester derivative of the heme from ROO that is liberated only after prolonged methanolysis. The bottom panel is the esterified, OCH_3 derivative of the porphyrin liberated from cytochrome c. The spectra are displayed in sections to emphasize the identity of the compounds. The chemical shifts are reported in the text. Both samples had impurities, mainly from solvent contaminants and column background, which appear as features from 5.3–5.6, 4.1–4.3, 2.8–3.0, and 2.3–2.4 ppm.

heme structure in two different environments, such as heme a and a_3 in mitochondrial cytochrome oxidase.

Finding Fe-uroporphyrin I associated with an enzyme is unprecedented to the authors' knowledge. Uroporphyrin, as the type III porphyrinogen, is the ubiquitous precursor for all known tetrapyrroles, but this is the first instance where any uroporphyrin appears in a mechanistically functional role. The nature of that function awaits further characterization of ROO. Uroporphyrin I has had no known physiologically significant role. It is well known in cases of porphyria or in mutants where the cosynthetase activity that produces the type III isomer has become impaired. The match of the pyridine hemochromogen and hemin spectra in Figs. 1 and 2 is strong evidence that it is indeed Fe-uroporphyrin, and not the free base, that is associated with the enzyme. The still mysterious 585-nm feature in ROO is not redox state dependent, but it is highly unlikely to be a free base porphyrin. Free base tetrapyrroles have multiple bands at least one of which will be above 600 nm (*8*), where the spectrum of ROO has no bands.

Scheme **2** is clearly related to heme c (Scheme **3**, R = Cys residues and polypeptide), but this does not mean ROO contains authentic heme c with thioether bonds. Recall that the hemes in ROO were extractable from the main polypeptide by acidic acetone. This is certainly not possible for normal cytochromes c where the acidic organic solvent precipitates the polypeptide without breaking the thioether bonds. The $-OCH_3$ groups in **2** probably come from methanol during the long acid-catalyzed solvolysis. In simple control experiments, it is possible to demonstrate that methanol-sulfuric acid precipitates cyt c, and the solid residue is then very resistant to further solvolysis of any sort. Weeks at room temperature are insufficient to liberate heme c from normal cyt c in this way. In strong aqueous acid (11% sulfuric acid), refluxing for 72 h does liberate heme from the polypeptide, but the main compound produced (Scheme **3** with R = $SCH_2CH(NH_3^+)COO^-$) still has the cysteine amino acids attached via the thioether bonds (*7*). We repeated this aqueous acid digestion experiment on horse cyt c and concentrated on the resultant products that could be extracted into ethylacetate after the hydrolysis. Only those products that had lost the zwitterionic cysteine residue would be expected to be extractable into ethylacetate. Extractable product was so obtained, but after esterification it was found to be a very complicated diastereomeric mixture of molecules containing $-CH(OH)CH_3$ and $-CH=CH_2$ groups. We could not isolate a single main product, and presumably all possible combinations were present, with racemization of the chiral centers. This is in stark contrast to the case of the ROO natural product where a single optically active product was obtained. Although indirect, these observations count against normal thioether bonds in the ROO heme. But there are other possible linkages that would also be susceptible to such hydrolysis. The stereochemistry of attachment is the same as in heme c, and the bonds are slowly acid labile. The attachment groups are not strongly electron delocalizing, and so the pyridine hemochromogen of **3** has an α maximum near 550 nm and is indistinguishable from that of Fe-uroporphyrin.

What are the possibilities for R in the heme of ROO? It cannot be the main polypeptide chain, since this precipitates in acetone. The flavin component has been identified as authentic FAD based on its spectrum and chromatographic

behavior. It might be a small, hydrophobic, acetone-soluble peptide that escaped detection during gel electrophoresis of ROO because of its size. Then the probability would increase that cysteine thioether bonds are also involved. The final obvious candidate is the mysterious 585-nm pigment.

Although the case is far from closed on the prosthetic groups of ROO, substantial progress has been made. There are two different hemes associated with ROO. One is the novel appearance of Fe-uroporphyrin, and the other is a heme related to heme c.

REFERENCES

1. SANTOS, H., FARELEIRA, P., XAVIER, A. V. L. C., LIU, M.-Y., AND LEGALL, J. (1993) *Biochem. Biophys. Res. Commun.* **195,** 551–557.
2. CHEN, L., LIU, M.-Y., LEGALL, J., FARELEIRA, P., SANTOS, H., AND XAVIER, A. V. (1993) *Biochem. Biophys. Res. Commun.* **193,** 100–105.
3. TIMKOVICH, R., CORK, M. S., AND TAYLOR, P. V. (1984) *J. Biol. Chem.* **259,** 1577–1585.
4. HARRIS III, W. F., BURKHALTER, R. S., LIN, W., AND TIMKOVICH, R. (1993) *Bioorg. Chem.* **21,** 209–220.
5. FUHRHOP, J.-H., AND SMITH, K. M. (1975) *in* Porphyrins and Metalloporphyrins (Smith, Ed.), pp. 803–804, Elsevier, Amsterdam.
6. LIN, W., HARRIS III, W. F., AND TIMKOVICH, R. (1994) *Bioorg. Chem.*, in press.
7. SLAMA, J. T., SMITH, H. W., WILLSON, C. G., AND RAPOPRT, H. (1975) *J. Am. Chem. Soc.* **97,** 6556–6562.
8. SMITH, K. M. (1975) *in* Porphyrins and Metalloporphyrins (Smith, Ed.), pp. 3–28, Elsevier, Amsterdam.