References and Notes

(1) This work was supported by U.S. Public Health Service SCOR Grant No. 1 P17 HL14147-04.
(2) Also the Department of Chemistry, Washington University, St. Louis, Mo. 63110.
(3) Department of Radiology (Nuclear Medicine), School of Medicine, University of California, Davis, Calif. 95616.
(4) D. Steinberg, M. Vaughn, C. B. Anfinsen, and J. Gorry, *Science*, **126**, 447 (1957).
(5) P. M. Yavorsky and E. Gorin, *J. Am. Chem. Soc.*, **84**, 1071 (1962).
(6) R. M. Campbell, D. P. Cuthbertson, C. M. Matthews, and A. S. McFarlane, *Int. J. Appl. Radiat. Isot.*, **1**, 66 (1956).
(7) L. Donato, G. Milhaud, and J. Sirchis, Ed., "Labeled Proteins in Tracer Studies", Eurotom, Brussels, 1966.
(8) "Radioisotopes in Medicine. In Vitro Studies", U.S. A.E.C. Publication CONF-671111 (1968).
(9) A. S. McFarlane, *J. Clin. Invest.*, **42**, 345 (1963).
(10) J. L. Oncley, *Fed. Proc., Fed. Am. Soc. Exp. Biol., Suppl.*, **16**, 3S (1957).
(11) U. Rosa, G. F. Pennisi, G. A. Scassellati, R. Bianchi, G. Federighi, L. Donato, C. A. Rossi, and C. Ambrosino, "Labeled Proteins in Tracer Studies", Eurotom, Brussels, 1966.
(12) W. L. Hughes, *Ann. N.Y. Acad. Sci.*, **70**, 3 (1957).
(13) F. C. Greenwood, W. M. Hunter, and J. S. Glover, *Biochem. J.*, **89**, 114 (1963).
(14) A. Appleby and R. P. Umfrid III, *J. Labelled Compd.*, **8**, 415 (1972).
(15) A. J. W. Brook and S. Housley, *J. Chromatogr.*, **41**, 200 (1969).
(16) M. K. Gaitonde and R. W. K. Nixey, *Anal. Biochem.*, **50**, 416 (1972).
(17) A. G. Perkins and A. B. Steven, *J. Chem. Soc.*, **89**, 802 (1906).
(18) M. Piattelli, E. Fattorusso, R. A. Nicolaus, and S. Magno, *Tetrahedron*, **21**, 3229 (1965).
(19) A. A. Patchett and B. Witkop, *J. Org. Chem.*, **22**, 1477 (1957).
(20) J. Cason, *Org. React.*, **4**, 305 (1948).
(21) C. E. M. Pugh and H. S. Paper, *Biochem. J.*, **21**, 1370 (1927).
(22) K. A. Krohn, L. Sherman, and M. J. Welch, *Biochem. Biophys. Acta*, **285**, 404 (1972).
(23) C. M. Lederer, J. M. Hollander, and I. Perlman, "Table of Isotopes", 6th ed, Wiley, New York, N.Y., 1967.
(24) T. A. Carlson and R. M. White, *J. Chem. Phys.*, **36**, 2883 (1962).
(25) T. A. Carlson and R. M. White, *J. Chem. Phys.*, **38**, 2075 (1963).
(26) T. A. Carlson, *J. Chem. Phys.*, **32**, 1234 (1960).
(27) Z. D. Draganic and I. G. Draganic, *J. Phys. Chem.*, **73**, 2571 (1969).
(28) H. Wagreich and J. M. Nelson, *J. Biol. Chem.*, **115**, 459 (1936).
(29) P. Neta and L. M. Dorfman, *Adv. Chem. Ser.*, **No. 81**, 222 (1971).
(30) B. D. Nefedev, E. N. Sunotova, and G. P. Syreischchikov, *Radiokhimiya*, **10**, 600 (1968).
(31) M. S. B. Munsan, *J. Am. Chem. Soc.*, **87**, 2332 (1965).
(32) "Handbook of Chemistry and Physics", 54th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1974.
(33) V. I. Vedeneyev, L. V. Gurvich, V. N. Kondrat'yev, V. A. Medvedev, and Ye. L. Frankevich, "Bond Energies, Ionization Potentials and Electron Affinities", E. Arnold, London, 1966.
(34) V. W. Jiang and M. J. Welch, unpublished results.
(35) "Handbook of Biochemistry", 2nd ed, Chemical Rubber Publishing Co., 1973.
(36) T. A. Carlson and R. M. White, *J. Chem. Phys.*, **38**, 2930 (1963).
(37) E. M. Diefallah, L. Stelter, and B. Diehn, *Radiat. Res.*, **44**, 273 (1970).
(38) T. Cacace, *Adv. Phys. Org. Chem.*, **8**, 79 (1970).

Porphyrin–Protein Bond of Cytochrome c. Structure of Porphyrin c

James T. Slama, Herman W. Smith, C. Grant Willson, and Henry Rapoport*

Contribution from the Department of Chemistry, University of California, Berkeley, California 94720.

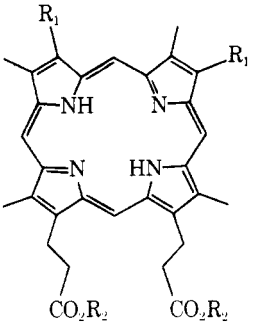
Received March 10, 1975

Abstract: Porphyrin c is the porphyrinic substance obtained from acid hydrolysates of cytochrome c. This substance, isolated from both yeast and horse heart cytochrome c, is shown to be 2,4-di(α-*S*-cysteinylethyl)deuteroporphyrin IX. A series of degradative, synthetic, and NMR studies are presented which conclusively establish the α-thioether linkage of the cysteine residues of the apoprotein to the 2- and 4-ethyl substituents of the porphyrin prosthetic group both in cytochrome c and in porphyrin c.

Cytochromes c are perhaps the most thoroughly investigated of all mitochondrial proteins, and the primary structures of cytochromes c from a wide variety of organisms have been elucidated.[1] The cytochromes c differ from most other hemoproteins in their covalent bonding of the iron–porphyrin prosthetic group to the apoprotein,[1a,2,3] whereas the bonding of protein to metalloporphyrin in other hemoproteins belongs to either the ligand or combined ligand-covalent types.[1a]

An appreciation of the detailed covalent bonding between porphyrin and peptide in cytochromes c has accrued over a 40-year period, beginning with the observation that acid hydrolysis of horse heart cytochrome c gave a homogeneous, amphoteric porphyrin fraction, porphyrin c.[4] This porphyrin c, upon treatment with hydrogen bromide in acetic acid, was converted to hematoporphyrin IX (**1**) which subsequently[5] was converted to mesoporphyrin IX (**2**).[5] These observations clearly established that porphyrin c belongs to the porphyrin III series.

Subsequent investigations,[6,7] including acid hydrolysis, demonstrated that porphyrin c was a dicysteinyl adduct of protoporphyrin IX and structure **3** was advanced. That is, the sulfhydryl groups of the cysteine residues were deduced to be attached to the 2,4-ethyl substituents of the porphyrin via thioether bonds, thus covalently joining amino acid to



1, R_1 = $CHOHCH_3$; R_2 = H
2, R_1 = CH_2CH_3; R_2 = H
3, R_1 = $CH(CH_3)SCH_2CH(NH_2)CO_2H$; R_2 = H
4, R_1 = $CHDCH_3$; R_2 = CH_3
5, R_1 = $CHDCH_2D$; R_2 = CH_3
7, R_1 = $CHBrCH_3$; R_2 = CH_3
8, R_1 = $CH_2CH_2SCH_2CH(NHCOCH_3)CO_2CH_3$; R_2 = CH_3
9, R_1 = $CH(CH_3)SCH_2CO_2CH_3$; R_2 = CH_3

porphyrin. However, the reservation was expressed[6b] that porphyrin c obtained by these hydrolytic methods may not reflect the original cytochrome thioether structure.[6b]

This question of cytochrome c thioether rearrangement

during hydrolysis presumably was obviated by the finding that metal salts cleave the porphyrin C–S bond of bovine heart cytochrome c to yield optically active hematoporphyrin derivatives.[8a] Although the mechanism of the cleavage is not known,[8b-d] this procedure and analogous ones[9] have been used to support the structure assigned to cytochrome c reconstituted from protoporphyrinogen and bovine heart apoprotein.[9b] Also, the structures of a porphyrin tetrapeptide,[9c] prepared from protoporphyrinogen and cysteinylglycylglycylcysteine, and a porphyrin pentapeptide were assigned on the basis of their mercuric sulfate cleavage product, hematoporphyrin.[10] An enzymatic approach[11] to this structural question has employed the C–S lyase of *Albizza lophanta*, known to cleave primary *S*-alkyl cysteines but not the corresponding secondary derivatives. When the synthetic porphyrin c prepared by fusion of cysteine with 2,4-di(α-bromoethyl)deuteroporphyrin was used as substrate, cleavage did not occur.[12]

The dimethyl esters of hematoporphyrin obtained from synthetic porphyrin c type compounds by silver salt cleavage, purified commercial hematoporphyrin, and isohematoporphyrin (**11**) have been compared.[9a] Neither the reported chromatographic behavior nor the ultraviolet-visible spectra of the isomeric α- and β-hydroxyethylporphyrins can definitively distinguish the isomers.

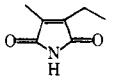
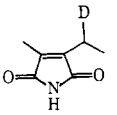
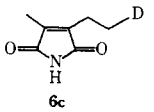
The structural and stereochemical integrity of the methods employed to degrade and synthesize cytochromes c, porphyrin c, and porphyrin peptides has yet to be conclusively demonstrated. That hydrolysis preserves the chirality of natural porphyrin c, a point crucial to interpretation of its optical properties, has not been established. The diagnostic value of silver or mercury salt cleavage methods remains untested for authenticated α- and β-linked porphyrin c type derivatives. That amino acid or protein chirality promotes asymmetric induction in metal-assisted C–S cleavage reactions persists as a viable explanation for the reported[7] optical activity of cytochrome c hematohemin ethers. It remains to be established if any porphyrin–cysteine adduct is a substrate for the C–S lyase of *Albizza lophanta*.

Two other studies directly on cytochromes c reflect on this question of α- or β-thioether linkage. Elegant X-ray studies[13a-d] on the eukaryotic cytochromes c have clearly established the shape of the molecule. However, the X-ray maps are insufficient to clarify the thioether question,[13e] although the X-ray structures are drawn α, as generally accepted. This is also true for the excellent X-ray crystallographic studies on the different, but related, prokaryotic cytochromes c, namely cytochrome c_{550} of *M. denitrificans*[13f] and cytochrome c_2 of *Rhodospirillum rubrum*.[13g]

Various cytochromes c and derived heme peptides have been examined by high-resolution proton magnetic resonance.[14-17] In several cases, specific high-field absorptions are attributed to the thioether bridge methyl groups.[5,16a,c] Assignments are based on integration, chemical shift, and spin-lattice relaxation time measurements.[15a] Such assignments, however reasonable, are still tentative. What is lacking is a demonstration that this method can distinguish an α-linked cytochrome from the β-type structure. Although the accumulation of supportive data makes assignment of the α-linkage highly likely, critiques of the individual investigations led us to seek methods which are completely unambiguous.

Since sodium amalgam reductive cleavage of cytochrome c is reported to give mesoporphyrin (**2**) in a 45–55% spectrophotometrically determined yield,[18] reduction in a deuterated solvent should label the mesoporphyrin at the position of C–S bond cleavage. Yeast cytochrome c was prepared and purified by described methods,[19] and the labile hydrogens were exchanged by repeated dissolution in and lyophilization from D_2O. The exchanged cytochrome was reduced with excess sodium amalgam in D_2O, the porphyrinogen was air oxidized, and the resulting mesoporphyrin was converted to the dimethyl ester.

Table I. Mass Spectra of Maleimides

Compd	% m/e 141	140	139	138	125	124
 **6a**	Nil	8.36	100	3.39	2.64	35.4
 **6b**	9.05	100	7.9	Nil	17.5	9.3
 **6c**	8.7	100	5.8	Nil	23.3	17.3
Maleimide from cytochrome[a]	8.8	100	6.1	Nil	17.5	10.0

[a] The data for the three synthetic compounds were obtained on an MS-9 spectrometer; that for the maleimide derived from cytochrome c was obtained on a CEC-21-103C instrument.

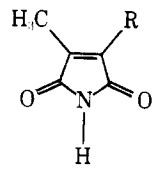
The NMR spectrum of the cytochrome mesoporphyrin exhibited a methyl doublet at δ 2.44 but the spin multiplicity of the methylene proton was unresolved in the 4.1 region. Also, the mesoporphyrin, having derived from the porphyrinogen, contained deuterium in the meso positions. NMR data alone suggested 2,4-di(α-deuterioethyl)deuteroporphyrin (**4**) as the product but neither integral nor spin multiplicity eliminated 2,4-di(α,β-dideuterioethyl)deuteroporphyrin (**5**). The molecular ion region in the mass spectrum of cytochrome c mesoporphyrin (*m/e* 594–600) indicated that the reduction and subsequent oxidation proceeded with incorporation of two to six deuterons. Mesoporphyrin (**2**) incorporated a maximum of four deuterons under identical reduction–oxidation conditions.

The labeled cytochrome mesoporphyrin was subjected to chromic acid oxidation[20] and the two resulting maleimides, 3-ethyl-4-methylmaleimide (**6**) and hematinic acid, were separated. Both NMR and mass spectral analysis of **6** indicated the incorporation of a single deuterium. The ratio of the 4-methyl singlet to the doublet methyl resonance of the 3-ethyl group was 1:1; the ratio of methylene to 3-methyl integrals was 1:3. Also, the mass spectrum of **6** exhibited a molecular ion at *m/e* 140 (Table I). However, the fragmentation pattern of the cytochrome c maleimide showed similar intensities for both *m/e* 124 and 125 ions, indicating that the benzylic type cleavage[21] resulted in loss of both CH_3 and CH_2D fragments. This observation could be interpreted to represent a proton–deuteron rearrangement associated with fragmentation. Analogous rearrangements are reported for butylthiophene[21] and even ethyl acetate.[22] In order to determine whether the M − 16 ion indeed arises as a result of such a rearrangement under electron bombardment or indicates an undetected isotopic mixture, we synthesized the specifically labeled maleimide isomers **6b,c**.

The synthesis of **6a** was accomplished from ethyl ethylacetoacetate,[20] and **6b,c** were prepared by alkylating acetoacetic ester with the appropriately labeled deuterioethyl iodides. The mass spectrum (Table I) of **6a** shows no appreciable M + 1 ion other than expected for natural abundance. The M − 15 fragment is a major ion (35.4%) and the M − 16 ion is very small. The mass spectra of the la-

Table II. NMR of Porphyrin c and Analogs

Compd	Protons of 2,4-ethyl groups, δ values in $CDCl_3$ (220 MHz) β	α
2,4-Di[α-(methoxycarbonylmethylthio)ethyl] deuteroporphyrin dimethyl ester (9)	2.40	6.04
2,4-Di[β-methoxycarbonylmethylthio)ethyl] deuteroporphyrin dimethyl ester (16)	Complex multiplet 3.2–4.6	
Synthetic porphyrin c (3)	2.54	6.0
Isoporphyrin c (10)	Complex multiplet 3–5	
Natural porphyrin c (in TFA)	2.59	6.00
Bis(*N*-acetyl)porphyrin c tetramethyl ester (8)	2.41	5.80
Bis(*N*-acetyl)isoporphyrin c tetramethyl ester (15)	Complex multiplet 3–4.6	



6a, R = CH_2CH_3
b, R = $CHDCH_3$
c, R = CH_2CH_2D

beled isomers **6b** and **6c** are quite similar and show comparable ion intensity at M − 16 and M − 15. Clearly a rearrangement occurred and the mass spectrum of the cytochrome maleimide is consistent with NMR data.

The results of these experiments demonstrate that the mesoporphyrin cleaved from cytochrome c by sodium amalgam in D_2O incorporated a single deuteron into each of the 2- and 4-ethyl substituents and, furthermore, that the deuterium is exclusively in the α position. It now was necessary to prove that the reductive cleavage of thioethers results in introduction of label at the original point of attachment of the S bond. The location of label was conclusively established by the above data. Was it the same as in the original structure? To answer this question we examined this reductive cleavage with both porphyrin c and isoporphyrin c.

Porphyrin c was first sought, and several published procedures for the preparation of porphyrin c by synthesis[7,9,12,23,24] and by hydrolysis of cytochrome c[7,6b,25] were attempted. In our hands, all of these procedures produced demonstrable mixtures, and a new countercurrent distribution purification procedure was developed for their separation. Bis(*N*-acetyl)porphyrin c tetramethyl ester (**8**) was prepared from 2,4-di(α-bromoethyl)deuteroporphyrin dimethyl ester (**7**) by treatment with *N*-acetyl-L-cysteine methyl ester in refluxing chloroform. Crude synthetic porphyrin c was obtained by the reported porphyrinogen procedure.[9] A modification of the sulfuric acid hydrolysis procedure[7] gave the natural samples from either yeast or horse heart cytochrome c.

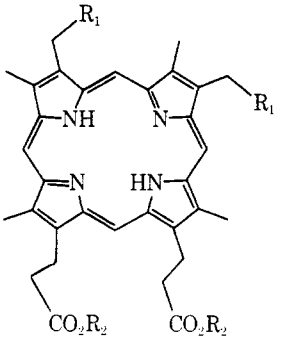
Pure samples of both natural and synthetic porphyrin c were obtained by sequential countercurrent distribution between aqueous pH 2 citrate buffer and 1-butanol. The effectiveness of this purification was demonstrated by the preparation of synthetic porphyrin c using [^{14}C]cysteine of known specific activity. The product isolated after two sequential countercurrent distributions of the crude material showed greater than 95% of the specific activity calculated for the bis(cysteine)protoporphyrin adduct.

In the preparation of yeast and horse heart porphyrin c, it was necessary to consider the possibility of rearrangement during the acid hydrolysis. After removal of iron from the cytochrome c, the porphyrin-protein was hydrolyzed in a tritiated sulfuric acid medium and the isolated porphyrin c, purified by two countercurrent distributions (100 tubes each), was found to have incorporated 0.38 solvent tritium atoms per molecule of porphyrin c. Mesoporphyrin (**2**) treated in the same way showed incorporation of 0.69 solvent tritiums per porphyrin.

An elimination–readdition mechanism involving the thioether bonds requires incorporation of two solvent protons per porphyrin. The low incorporation level of tritium into porphyrin c indicated that such a mechanism was inoperative. The results of the mesoporphyrin control experiment, consistent with those of the porphyrin c experiment, establish that the label incorporated into porphyrin c was the result of nonspecific electrophilic incorporation rather than any rearrangement or racemization mechanism. Analogous electrophilic attack at the porphyrin meso positions has been described.[26,27] Thus, we conclude that the porphyrin–cysteine linkage in porphyrin c faithfully reflects the attachment of the heme to the cytochrome c peptide chain.

We can also conclude from the labeling experiment that little or no racemization at the α position of the cysteinyl residues occurred during the hydrolysis. This observation is consistent with previous studies which showed that cysteine requires a disulfide linkage for rapid racemization under acidic conditions.[28] The model compound, *S*-benzylcysteine, was racemized only to the extent of ~3% in refluxing 20% sulfuric acid in 24 hr.

We approached the synthesis of isoporphyrin c (**10**) via



10, R_1 = $CH_2SCH_2CH(NH_2)CO_2H$; R_2 = H
11, R_1 = CH_2OH; R_2 = H
12, R_1 = $CH(OCH_3)_2$; R_2 = CH_3
13, R_1 = CHO; R_2 = CH_3
14, R_1 = CH_2Br; R_2 = CH_3
15, R_1 = $CH_2SCH_2CH(NHCOCH_3)CO_2CH_3$; R_2 = CH_3
16, R_1 = $CH_2SCH_2CO_2CH_3$; R_2 = CH_3

elaboration of a preformed porphyrin nucleus. Initial attempts via radical thioylation or Michael-like addition to protoporphyrin were unsuccessful. We then turned to isohematoporphyrin [2,4-di(β-hydroxyethyl)deuteroporphyrin (**11**)] as a precursor. This compound has been prepared via thallium trinitrate mediated oxidative rearrangement of protoporphyrin.[29] Following this procedure, protoporphyrin dimethyl ester was converted to isohematoporphyrin dimethyl ester via the dimethyl acetal **12** and the aldehyde **13**. Alternatively, we have found that **11** can be prepared directly from protoporphyrin by treatment with diborane followed by hydrogen peroxide. Formation of the β,β′-dibromo ester **14** proceeded smoothly,[30] and when the dibromoporphyrin was treated with the lithium thiolate salt of *N*-acetyl-L-cysteine methyl ester in THF, bis(*N*-acetyl)isoporphyrin c tetramethyl ester (**15**) was isolated in 40% yield.

Spectroscopic evidence for the α linkage in porphyrin c has accrued from the NMR spectra of the porphyrin c iso-

mers and various model compounds. These spectra are presented in part in Table II where explicit assignments of the ethyl protons geminal and vicinal to the sulfur are listed. In each pair of isomers studied, the methyl (vicinal) protons of the α isomer resonate at least at 1-ppm higher field than any absorption which occurs in the β isomer. The α isomers all exhibit a characteristic 6-proton resonance between δ 2 and 3. This signal has been assigned, on the basis of double resonance experiments, to the methyl groups of 2- and 4-ethyl substituents.

The NMR spectrum of natural porphyrin c (in TFA at 220 MHz) twice purified by countercurrent distribution is consistent with the α-thioether linkage as evidenced by the six-proton β-methyl absorption at δ 2.59, as well as the two-proton methine absorption at 6.0. Similar signals are present in the NMR of synthetic porphyrin c[31] but completely absent from the spectrum of isoporphyrin c (Figure 1). Although the chemical shifts of the two methylene protons of the isoporphyrin c cannot be assigned due to complexity, it is clear that these signals must occur in the region between 3 and 5 ppm. Similar differences between α- and β-linked thioethers are observed in the α- and β-bis(thioglycolate)protoporphyrin adducts. Given the clear differences in the NMR spectra of α- and β-linked isomers, it is possible unequivocally to assign natural porphyrin c to the α series on the basis of the NMR data.

Treatment of bis(*N*-acetyl)porphyrin c tetramethyl ester with sodium amalgam under the conditions used to cleave cytochrome c gave mesoporphyrin. However, under identical conditions the β isomer **15** gave a porphyrin mixture which contained no mesoporphyrin. Thus both the sodium amalgam and hydrolytic methods of degrading α-linked cytochromes c proceed with integrity, and the products reflect the structure of the α-thioether in the original cytochrome c.

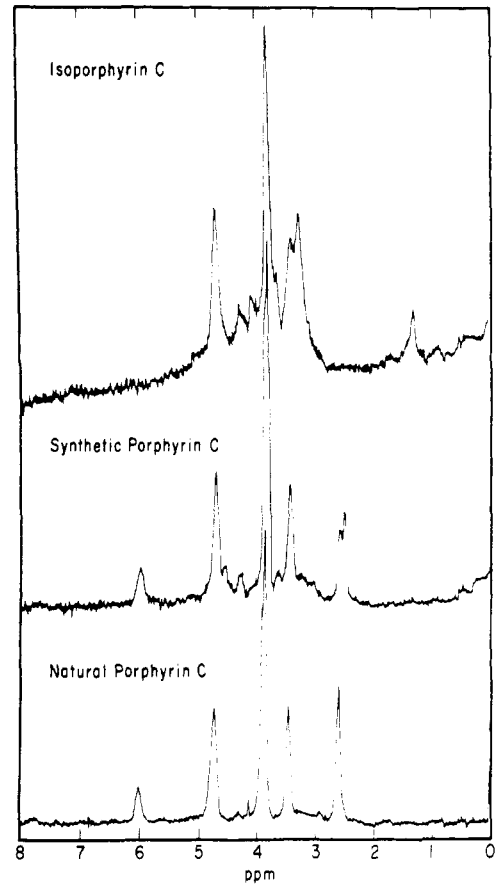


Figure 1. NMR of synthetic porphyrin c, isoporphyrin c, and natural porphyrin c.

Experimental Section[32]

Horse heart cytochrome c was obtained from Calbiochem (A grade, equine heart, assay 88.3%).

Yeast Cytochrome c. Fleischmann's pressed baker's yeast (27.2 kg) was extracted, purified, and analyzed essentially as described[19] except that a step, rather than linear, gradient was used in the final purification; the yield was 70–80 mg of cytochrome c/kg of yeast.

Isolation of Natural Porphyrin c from Hydrolysis of Cytochrome c. Cytochrome c (3.0 g, horse heart) was dissolved in 750 ml of 0.05 *N* NaOH. To this solution was added 6.3 g of $Na_2S_2O_4$, followed by the rapid addition of 96 ml of 96% w/w sulfuric acid; the solution was brought to a boil and boiled for 5 min and then centrifuged. The clarified supernatant was added to 7.5 kg of 20% w/w sulfuric acid, previously deoxygenated by boiling overnight under a nitrogen blanket, and the solution was brought to reflux and refluxed for 27 hr all under nitrogen, followed by cooling to room temperature and standing overnight. Neutralization by the addition of alkali (1.36 kg of NaOH in 1.5 l. of H_2O) to pH 3.1 was followed by passage through a column of carboxylic acid exchange resin (Biorex-70, 50–100 mesh, H^+ form, 8 × 1 in.). Adsorbed porphyrin was washed with 500 ml of 0.1 *M* citrate buffer, pH 3, 500 ml of 0.01 *M* citrate buffer, pH 3, and 500 ml of distilled water. Porphyrin was eluted with 30% pyridine in water, and solvent was removed by lyophilization.

Two batches of crude porphyrin c, prepared as described above, were combined for further purification by countercurrent distribution. Citrate buffer was prepared by dissolving 42 g of citric acid·H_2O in 400 ml of 1 *N* NaOH, diluting to 3 l. with distilled water, and adjusting the pH to 2.0 with concentrated HCl. The buffer was equilibrated by shaking with an equal volume of 1-butanol overnight, and the pH of the aqueous buffer phase was 2.2–2.3 after equilibration.

A 100-transfer countercurrent distribution with a 10-ml lower phase was then performed on the material isolated from the hydrolysis, using an automatic Craig-type instrument. Three fractions were observed: the first in tubes 10–22, the second in tubes 30–60, and the last in tubes 70–100. Tubes 30–60, fraction II, were combined, all the material was driven into the lower phase via addition of cyclohexane to the butanol, the pH was adjusted to 3.5, and porphyrin was adsorbed on a column of Biorex-70 (H^+ form). After washing with water, porphyrin was eluted with 30% pyridine–water and lyophilized, leaving 107 mg of crude porphyrin c. This was redissolved in lower phase buffer and submitted to another CCD purification under identical conditions, leaving 61 mg of porphyrin c after lyophilization.

The powdery residue was taken up in 22 ml of 1 *N* HCl and filtered, the pH was adjusted to 4.0 using 10 *N* NaOH, the suspension was filtered through a millipore filter after cooling for several hours, and the precipitate was washed with water and dried over magnesium perchlorate in vacuo to leave 39.5 mg (11% yield) of porphyrin c as a hygroscopic, amorphous powder; uv [1 *N* HCl, λ_{max} nm ($\epsilon \times 10^{-4}$)] 406 (36.0), 552 (1.60), 594 (0.58).

Anal. Calcd for $C_{40}H_{48}N_6O_8S_2$: C, 59.7; H, 6.0; N, 10.4. Found: C, 59.3; H, 6.3; N, 9.8.

Hydrolysis of yeast cytochrome c, performed in the same way, gave porphyrin c identical chromatographically and spectrophotometrically with that from horse heart.

Hydrolysis in a tritiated medium was carried out exactly as described using 1.0 g of cytochrome c and adding 279 mCi of tritium, as T_2O, to the hydrolysis medium. Porphyrin c was obtained in 11 mg yield. It was shown to be free of labile tritium by solution in 30% pyridine–water, lyophilization, and counting of the lyophylate, which contained no radioactivity. The specific activity of the sulfuric acid hydrolysis medium, per atom of hydrogen, was 1.20 μCi/mmol; that of the porphyrin c was 1250 dpm/mg or 0.457 μCi/mmol. Therefore, incorporation of tritium from the solvent into the porphyrin c was 0.457/1.20 = 0.38 H atom per molecule of porphyrin.

Tritiation of Mesoporphyrin. Mesoporphyrin IX dimethyl ester was prepared by catalytic hydrogenation of protoporphyrin IX dimethyl ester over PtO_2 and was purified by chromatography on alumina and rechromatographed on silica gel, followed by crystallization. A sample was examined by emission spectrographic anal-

ysis and no platinum was detected (that is, less than 0.07% by weight was present or 0.05 mol % of the platinum-mesoporphyrin complex). The mesoporphyrin IX dimethyl ester (59.4 mg) was added to 100 g of 20% (w/w) sulfuric acid containing 5.6 mCi of tritiated water, the solution was refluxed under a nitrogen blanket for 25 hr and then cooled, 15.4 g of solid NaOH was added, and the precipitated porphyrin was recovered by passing the solution through a short Biorex 70 column, washing with 250 ml of distilled water, and eluting with 30% pyridine-water.

Lyophilization left 35 mg of mesoporphyrin which was suspended in 10 ml of 95% ethanol, 0.5 ml of 2 *N* KOH, and 5 ml of H_2O, and with vigorous stirring was added 1.1 g of K_2CO_3 followed by 2.9 g of triethyloxonium fluoroborate in small portions over a period of 30 min. After addition was complete, the suspension was stirred 30 min, poured into 100 ml of distilled water, and extracted with three 25-ml portions of ethyl acetate. The combined ethyl acetate was washed with 25 ml of 1 *N* NH_4OH, two 50-ml portions of water, and saturated aqueous sodium chloride. Drying over magnesium sulfate and evaporating gave 48 mg of residue which was purified on a silica gel column (10 g of silica gel 60, 1 × 17 cm, eluting with $CHCl_3$–0.75% CH_3OH), collecting the first, fast-moving band. Crystallization from benzene-methanol gave 29 mg of mesoporphyrin diethyl ester. This was washed by dissolving in $CHCl_3$ or ethyl acetate (10 ml), adding 10 ml of 1 *N* HCl, equilibrating, neutralizing the acid with 10 *N* NaOH, and separating the phases. The process was repeated four times; then solvent was evaporated and the mesoporphyrin diethyl ester was crystallized. The washing procedure was repeated as above two more times. The specific activity of the hydrolysis medium was 0.56 μCi/mg-atom of H; the specific activity of the mesoporphyrin diethyl ester was 0.385 μCi/mmol; therefore, the incorporation was 0.385/0.56 = 0.69 H atoms per mesoporphyrin molecule.

Optical Stability of *S*-Benzylcysteine. *S*-Benzylcysteine (1.0 g, $[\alpha]^{20}_{589}$ 23.7°)[33] was dissolved in 100 ml of 20% H_2SO_4 (w/w) water solution, previously deoxygenated by reflux under a N_2 blanket, and the solution was refluxed for 24 hr. Amino acid analysis at this time revealed no new ninhydrin-positive components. The acid was neutralized with 48 ml of 10 *N* NaOH, the pH was adjusted to 6, and the precipitated amino acid was recrystallized from 40 ml of boiling water to give 630 mg of *S*-benzylcysteine: $[\alpha]^{20}_{589}$ 21.1°. Using an identical procedure with that above, except that 5.6 mCi of ^3H_2O was added, the recovered *S*-benzylcysteine showed incorporation of 0.019/0.59 or 0.034 H atom per cysteine. Thus 3.4% of the cysteine was racemized.

Sodium Amalgam Reduction of Cytochrome c. Exchangeable protons in cytochrome c were replaced with deuterons by repeated (four times) solution in D_2O and lyophilization. The deuterated cytochrome c so obtained (0.49 g, 0.039 mmol) in 99.8% deuterium oxide (25 ml) at 55° was treated with 6% sodium amalgam (40 g) in 5-g aliquots during 40 min, at which point the cytochrome color was discharged. Stirring was continued for 45 min, the colorless solution decanted, unreacted amalgam washed with water, and the combined aqueous solution acidified with acetic acid and extracted with ethyl acetate. The extract was exposed to air for 12 hr with light exclusion, dried, and evaporated, leaving a residue which was esterified with 18% methanolic hydrogen chloride (150 ml) for 4 hr, evaporated, and diluted with CH_2Cl_2. The residue from evaporation of the sodium bicarbonate washed CH_2Cl_2 was chromatographed on alumina; elution with 1:1 benzene-methylene chloride gave the mesoporphyrin fraction which crystallized from methylene chloride-methanol to yield 7 mg (0.012 mmol, 30%) of 2,4-di([α-$^2H_1$]ethyl)deuteroporphyrin dimethyl ester (**4**): mp 217°, partially deuterated at the meso positions; NMR ($CDCl_3$) δ 1.83 (d, J = 7 Hz, 6, CH_3CH), 3.27 (t, J = 8 Hz, 4, $CH_2CH_2CO_2R$), 3.50–3.77 (m, 18, CH_3), 4.20 (m, CH_2CH_3), 4.40 (t, J = 8 Hz, 4, $CH_2CH_2CO_2R$), 10.1 (s, 1.25, meso); mass spectrum 601 (34.9%, $C_{35}{}^{13}CH_{34}D_6O_4N_4$), 600 (87.0, $C_{36}H_{34}D_6O_4N_4$), 599 (100, $C_{36}H_{35}D_5O_4N_4$), 597 (28.2), 596 (10.7).

Reductive cleavage with Na–Hg was carried out in the same manner with porphyrin c, isoporphyrin c, and their bis(*N*-acetyl) tetramethyl esters.

Methyl[α-$^2H_1$]ethylmaleimide from 2,4-Di([α-$^2H_1$]ethyl)deuteroporphyrin Dimethyl Ester (4). The deuterated mesoporphyrin dimethyl ester **4** (17 mg, 0.029 mmol) in 50% (v/v) sulfuric acid (2 ml) was hydrolyzed and oxidized, and the products were purified by reported[20] procedures. The crystalline methyl[α-$^2H_1$]ethylmaleimide, mp 64° (lit.[20] mp 68°), was identical with authentic methylethylmaleimide: NMR ($CDCl_3$) δ 1.18 (d, J = 7 Hz, 3, CH_3CH), 2.00 (s, 3, CH_3), 2.35 (m, 1, $CHCH_3$); mass spectrum 141 (8.8), 140 (100), 139 (6.1), 125 (17.5), 124 (10.0), 112 (3.4), 111 (6.0), 110 (1.0), 97 (12.9), 96 (4.7), 69 (19.5), 68 (28.4), 67 (9.5), 66 (4.7), 54 (16.2), 53 (7.5).

[1-$^2H_1$]Ethyl Iodide. [1-$^2H_1$]Acetaldehyde was prepared from 2-methyl-1,3-dithiane[34] and the product was collected by passing a slow current of nitrogen through the hydrolysis reaction and into a −80° trap. The contents of the trap (14 ml) were distilled slowly into a mixture of sodium borohydride (5.0 g) in 75 ml of diglyme and 10 ml of water at 0° and the resulting alcohol was isolated by distillation. The fraction boiling at 75–90° was treated with 2 g of phosphorus and 24 g of iodine to give 21.8 g (44% from 2-methyl-2-[$^2H_1$]-1,3-dithiane) of [1-$^2H_1$]ethyl iodide: NMR (neat) quartet of triplets at δ 3.15 (J_q = 8.0 Hz, J_t = 1.50 Hz), doublet of triplets at 1.80 (J_d = 8.0 Hz, J_t = 1.75 Hz); mass spectrum, isotopic purity greater than 98% based on $(M^+ - 1)/[M^+ + (M^+ - 1)]$.

Ethyl [1-$^2H_1$]ethylacetoacetate was prepared as described for the *n*-butyl analog.[35] The product was distilled through a 10-cm Vigreux column to give 89% yield: bp 77–80° (10 Torr); NMR ($CDCl_3$) δ 4.19 (q, 2, J = 7.0 Hz), 3.31 (d, 1, J = 7.0 Hz), 2.20 (s, 3), 1.83 (m, 1), 1.26 (t, 3, J = 7.0 Hz), 0.94 (d, , J = 7.0 Hz).

Methyl[1-$^2H_1$]ethylmaleimide. Methyl[1-$^2H_1$]ethylmaleic anhydride was prepared as described for the nonisotopically labeled compound:[20] NMR ($CDCl_3$) δ 2.50 (q, 1, J = 8.0 Hz), 2.00 (s, 3), 1.10 (d, 3, J = 8.0 Hz); mass spectrum *m/e* 141 (100), 126 (9), 125 (4.4).

This anhydride was converted to **methyl[1-$^2H_1$]ethylmaleimide** by heating with urea in the standard way:[20] NMR ($CDCl_3$) δ 2.42 (q, 1, J = 8.0 Hz), 198 (s, 3), 1.10 (d, 3, J = 8.0 Hz). Mass spectrum *m/e* 124, 125 with relative abundance 9.3, 17.5 at 70 eV and 10.8, 14.7 at 15 eV, respectively.

[2-$^2H_1$]Ethyl Iodide.[36] 2-Iodomercuriethyl benzyl ether[37] (72 g, 0.15 mol) was treated with 2% sodium amalgam (100 g), in 200 ml of D_2O. The resulting mixture was vigorously stirred under nitrogen at room temperature for 20 hr, an additional 50 g of 5% amalgam was added and stirring was continued for 4 hr; another 25 g of 5% amalgam was then added and the mixture was stirred overnight. The mercury was removed, CH_2Cl_2 was added, and the aqueous phase was separated and washed with three 100-ml portions of CH_2Cl_2. The combined organic extract was dried, the solvent was evaporated, and the residue, dissolved in hexane, was filtered through a short column of alumina (neutral, activity I) to remove benzyl alcohol. Evaporation of the hexane left 5.25 g, 23% yield, of pure **benzyl [2-$^2H_1$]ethyl ether:** NMR (CCl_4) δ 7.25 (s, 5), 4.4 (s, 2), 3.4 (t, 2, J = 7.4 Hz), 1.2 (2, t of t, J_{H-H} = 7.4 Hz, J_{H-D} = 1 Hz); mass spectrum *m/e* 135 (1.3), 136 (90), 137 (100), 138 (11).

To all of the benzyl [2-$^2H_1$]ethyl ether prepared above in 25 ml of decalin was added 1 g of $NaBH_4$. To this stirred suspension was added 7 g (0.025 mol) of iodine and the resulting mixture was heated and stirred at 110° while a slow nitrogen stream was swept over the reaction mixture into a −80° trap. After 18 hr, the liquid contained in the trap was washed with dilute $NaHSO_3$ and dried over molecular sieves to give 1.91 g (30%) of [2-$^2H_1$]ethyl iodide; mass spectrum *m/e* 158 (2.8), 157 (100), 156 (1.95), which corresponds to >98% isotopic purity.

Methyl[2-$^2H_1$]ethylmaleimide (6c) was prepared as described for the isomer: NMR ($CDCl_3$) δ 2.40 (t, 2, J = 8 Hz), 2.0 (s, 3), 1.20 (2, tt, J_{H-H} = 8 Hz, J_{H-D} = 1 Hz); mass spectrum *m/e* 141 (8.7), 140 (100), 139 (5.8), 125 (23.3), 124 (17.3).

Hematoporphyrin Dibromide Dimethyl Ester (7) Dihydrobromide. Hematoporphyrin dimethyl ester (1.21 g, 2.0 mmol) in 15 ml of glacial acetic acid was cooled at 0° and saturated with HBr. The resulting solution was stripped of solvent in vacuo and the residue was dried overnight at 0.5 Torr over KOH. The crude dihydrobromide salt was recrystallized from methylene chloride–cyclohexane to give 1.275 g (70%) of purple crystals. Attempts to isolate the free base were not successful and invariably resulted in elimination to yield protoporphyrin: NMR ($CDCl_3$) δ 11.0 (m, 3, meso), 10.03 (s, 1, meso), 6.59 (m, 2, -CHBr-), 4.35 (t, 4, J = 6.8 Hz, propionate CH_2), 3.7–3.1 (m, 24, ring and ester CH_3), 2.54 (d, 6, J = 7.0 Hz, $-CHBrCH_3$).

Bis(*N*-acetyl)porphyrin c Tetramethyl Ester (8). To hematoporphyrin dibromide dimethyl ester (**7**) dihydrobromide (942 mg, 1

mmol) dissolved in 25 ml of methylene chloride was added *N*-acetyl-L-cysteine methyl ester (885 mg, 5 mmol) and 10 ml of propylene oxide. The solution was refluxed overnight, cooled, and washed with saturated aqueous bicarbonate and water. The organic layer was dried over sodium sulfate, the solvent was evaporated, and the residue was chromatographed on activity III neutral alumina (benzene–ethyl acetate, 1:1) and the two slow moving bands were isolated. These were then each rechromatographed on kieselgel (benzene–ethyl acetate, 1:1). The slowest moving band was characterized as bis(*N*-acetyl)porphyrin c tetramethyl ester (**8**): NMR ($CDCl_3$) δ 10.6 (m, 2, meso), 10.1 (m, 2, meso), 6.3 (m, 2, NH), 5.8 (m, 2, $SCHCH_3$), 5.8 (m, 2, $CH_3CONHCHCOOCH_3$), 4.3 (m, 4, $CH_2CH_2COOCH_3$), 3.9–3.0 (unresolved envelope), 2.45 (d, 6, J = 7 Hz, $SCHCH_3$), 1.90 (s, 6, CH_3CON); mass spectrum *m/e* 767, 647, 558.

Anal. Calcd for $C_{48}H_{60}O_{10}N_6S_2$: C, 61.0; H, 6.4; N, 8.9. Found: C, 61.4; H, 6.2; N, 8.6.

2,4-Di[(α-methoxycarbonylmethylthio)ethyl]deuteroporphyrin Dimethyl Ester (9). To hematoporphyrin dibromide dimethyl ester (**7**) dihydrobromide (0.53 g, 0.58 mmol) dissolved in 5 ml of methylene chloride was added 2 ml of thioglycolic acid. The solution was stirred in the dark at room temperature for 22 hr, the solvent was evaporated, the residue was dissolved in 20 ml of water and neutralized to pH 4 with concentrated NH_4OH, and the precipitate was removed by centrifugation. The resulting brown solid was dissolved in dilute ammonium hydroxide and reprecipitated at pH 4 with acetic acid. The precipitate was dissolved in 50 ml of 20% (w/w) methanolic HCl and stirred at 0° for 5 hr. The solvent was evaporated; the residue was dissolved in methylene chloride, washed with bicarbonate and water, and dried over Na_2SO_4. Evaporation of the methylene chloride left a residue which was chromatographed on activity III, neutral alumina using 1:1 benzene–ethyl acetate. The first, and major, fraction was collected and evaporated to leave a red-brown powder which slowly crystallized from 2-propanol: 180 mg; 40% yield; NMR ($CDCl_3$) δ 10.62 (s, 1, meso), 10.53 (s, 1, meso), 10.20 (s, 1, meso), 10.10 (s, 1, meso), 6.20 (m, 2, $SCHCH_3$), 4.35 (t, 4, $CH_2CH_2CO_2CH_3$), 3.3–3.8 (m, 24, CH_3, CH_3O), 3.20 (s, 4, $CH_2CO_2CH_3$), 2.44 (d, 6, J = 6 Hz, $SCHCH_3$); the doublets at 2.44 collapse when the multiplet at 6.20 is irradiated.

Anal. Calcd for $C_{42}H_{50}N_4O_8S_2$: C, 62.8; H, 6.3; N, 7.0; S, 8.0. Found: C, 63.1; H, 6.0; N, 6.9; S, 7.8.

2,4-Di(2-hydroxyethyl)deuteroporphyrin Dimethyl Ether (Isohematoporphyrin Dimethyl Ester) (11). (a) This compound was prepared via the acetal **12** as described:[29] mp 225–226° (lit.[29] mp 225–226°); uv ($CHCl_3$) λ_{max} 498, 533, 569, 623 nm; mass spectrum *m/e* 626 (100), 595 (48).

Anal. Calcd for $C_{36}H_{42}O_6N_4$: C, 69.0; H, 6.8; N, 8.9. Found: C, 68.7; H, 6.8; N, 8.8.

(b) To protoporphyrin dimethyl ester (590 mg, 1 mmol) dissolved in 70 ml of THF and cooled to 15° was added 4 mmol of B_2H_6 in THF all at once. While the temperature was maintained at 15°, 4 ml of 6 *N* sodium hydroxide was added slowly, followed by 4 ml of 30% hydrogen peroxide. The reaction was stirred for 20 min, acidified with excess acetic acid, poured onto solid sodium bicarbonate, diluted with twice its volume of water, and extracted with chloroform. The chloroform extracts were combined and dried over sodium sulfate, and the solvent was removed in vacuo. The residue was chromatographed on 50 g of silica gel using hexane–acetone to give 230 mg of product as the slowest running band (R_f 0.25, 1:1 hexane–acetone). The isohematoporphyrin dimethyl ester (**11**) was crystallized from pyridine–ether and was identical by uv, mass spectrum, TLC, and NMR to the product prepared above from the acetal. The product contained no detectable hematoporphyrin as shown by TLC on silica in 1:1 hexane–acetone which completely resolves hemato- and isohematoporphyrin.

2,4-Di(2-chloroethyl)deuteroporphyrin Dimethyl Ester. This compound was prepared in 83% yield from **11** as described.[30] The bromo derivative **14** was prepared in an identical fashion using thionyl bromide in 69% yield: NMR ($CHCl_3$) (chloro and bromo distinguishable) δ 9.80 (s, 1, meso), 9.50 (s, 2, meso), 9.21 (s, 1, meso), 3.6–4.3 (m, 8, CH_2CH_2Cl), 3.6–2.8 (unresolved envelope); uv $\lambda_{max}^{CHCl_3}$ 623, 568, 533, 499; mass spectrum (chloro) *m/e* 664 (68), 662 (100), 628 (45), 626 (32), 590 (50); mass spectrum (bromo) *m/e* 754 (9), 752 (17), 750 (8), 672 (65), 670 (50), 590 (100).

Bis(*N*-acetyl)isoporphyrin c Tetramethyl Ester (15). To THF (5 ml) cooled to −80° was added methanol (200 μl) followed by 380 μl of 2.67 *M* butyllithium in hexane. The resulting solution was treated with 311 mg (1.76 mmol) of *N*-acetyl-L-cysteine methyl ester in 3 ml of THF and warmed to room temperature, 133 mg (0.176 mmol) of the β,β′-dibromoethylporphyrin ester **14** was added, and the resulting solution was heated at reflux for 0.5 hr. The reaction mixture was cooled, diluted with water, and extracted with chloroform, and the chloroform extract was washed with water, dried, and evaporated to dryness. The residue was chromatographed on 60 g of kieselgel, developing the column with methylene chloride and eluting the product with 20% acetone in methylene chloride and crystallizing it from 2-propanol: yield 108 mg of **15**; NMR ($CHCl_3$) δ 9.85 (m, 4, meso), 6.38 (m, 2, NH), 4.82 (m, 4, $SCH_2CH<$), 4.6–3.0 (unresolved envelope), 1.90 (s, 6, CH_3CON).

Anal. Calcd for $C_{48}H_{60}O_{10}N_6S_2$: C, 61.0; H, 6.5; N, 8.9. Found: C, 61.3; H, 6.4; N, 8.6.

Isoporphyrin c (10). A solution of 20% (w/w) sulfuric acid (2300 ml) was degassed by boiling under N_2 for 2.5 hr, 42 mg of bis(*N*-acetyl)isoporphyrin c tetramethyl ester (**15**) was added, and the solution was refluxed for 16 hr, cooled to 50°, and neutralized to pH 3 with concentrated NaOH. Neutralization was conducted with cooling such that the temperature never exceeded 70°. The pH 3 solution was passed through a Biorex 70 column (H^+ form, 80–100 mesh) at 40°, the column was washed with 4 l. of H_2O, and the porphyrin was eluted with 30% pyridine–water. The bright red eluate was lyophilized and the residue was purified by countercurrent distribution between pH 2 citrate and 1-butanol. The purple band at K_D = 1 was isolated to leave 19 mg of pure material: NMR (TFA) δ 4.9–3.0 (unresolved envelope), 2–3 (no absorption).

2,4-Di[β-methoxycarbonylmethylthio)ethyl]deuteroporphyrin Dimethyl Ester (16). Protoporphyrin (0.25 g, 0.435 mmol) in thioglycolic acid (6 ml) was heated for 18 hr at 95°. The solution was diluted with ice–water, treated with sodium acetate to pH 5, and kept at 4° for 12 hr. The semicrystalline precipitate (0.32 g) was esterified with 18 wt % methanolic hydrogen chloride, and the concentrated solution was diluted with methylene chloride and washed with 5% sodium bicarbonate and saline solutions. Chromatography of the residue from evaporation of the organic phase gave, after methylene chloride elution from alumina and crystallization (CCl_4), 27.5 mg (0.034 mmol, 8%) of **16**: NMR ($CDCl_3$) δ 3.18 (m, $CH_2CO_2CH_3$, CH_2S), 3.62 (m, CH_3, CH_3O), 10.1 (m, meso).

Anal. Calcd for $C_{42}H_{50}N_4O_8S_2$: C, 62.8; H, 6.3; N, 7.0. Found: C, 62.8; H, 5.9; N, 7.0.

References and Notes

(1) (a) E. Margoliash and A. Shejter, *Adv. Protein Chem.*, **21**, 113 (1966); (b) R. Dickerson, *J. Mol. Evol.* **1**, 26 (1971).
(2) D. Hulquist and M. Morrison, *J. Biol. Chem.*, **238**, 2843 (1963).
(3) J. Barrett and M. Kamen, *Biochem. Biophys. Acta*, **50**, 573 (1961).
(4) R. Hill and D. Keilin, *Proc. Roy. Soc., Ser. B*, **107**, 286 (1930).
(5) K. Ziele and F. Reuter, *Z. Physiol. Chim.*, **221**, 101 (1933).
(6) (a) H. Theorell, *Enzymologia*, **6**, 88 (1939); (b) H. Theorell and A. Akesson, *J. Am. Chem. Soc.*, **63**, 1804 (1941).
(7) K. Ziele and H. Meyer, *Z. Physiol. Chim.*, **262**, 178 (1939).
(8) (a) K. Paul, *Acta Chem. Scand.*, **4**, 239 (1950); (b) K. Paul, *ibid.*, **5**, 389 (1951); (c) H. E. Davenport, *Proc. Roy Soc., Ser. B*, **139**, 327 (1952); (d) C. Rimington, W. H. Lockwood, and R. V. Belcher, *Clin. Sci.*, **35**, 211 (1968).
(9) (a) S. Sano, N. Nanzyo, and C. Rimington, *Biochem. J.*, **93**, 270 (1964); (b) S. Sano and K. Tanaka, *J. Biol. Chem.*, **239**, 3109 (1964); (c) S. Sano, K. Ikada, and S. Sakakibara, *Biochem. Biophys. Res. Commun.*, **15**, 284 (1964); (d) M. Momenteau and B. Loock, *Biochem. Biophys. Acta*, **343**, 535 (1974).
(10) E. Becker, R. Bradley, and C. Watson [*J. Am. Chem. Soc.*, **83**, 3743 (1961)] have speculated that a pure sample of hematoporphyrin has never been obtained.
(11) S. Hansen, A. Kjaer, and S. Schwimmen, *C. R. Trav. Lab. Carlsberg, Ser. Chim.*, **31**, 193 (1959).
(12) J. Nielands and H. Tuppy, *Biochem. Biophys. Acta*, **38**, 351 (1960).
(13) (a) R. E. Dickerson, M. L. Kopka, J. Weinzierl, J. Varnum, D. Eisenberg, and E. Margoliash, *J. Biol. Chem.*, **242**, 3015 (1967); (b) R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kalai, L. Samson, A. Cooper, and E. Margoliash, *ibid.*, **246**, 1511 (1971); (c) T. Takano, O. B. Kallai, R. Swanson, and R. E. Dickerson, *ibid.*, **248**, 5234 (1973); (d) K. Tanaka, T. Yamane, T. Tsukihara, T. Ashida, and M. Kakudo, *J. Biochem.*, **77**, 147 (1975); (e) K. Raymond and D. Templeton, personal communications; (f) R. Timkovich and R. E. Dickerson, *J. Mol. Biol.*, **79**, 39 (1973); (g) F. R. Salemme, S. T. Freer, Ng. H. Xuong, R. A. Alden, and J. Kraut, *J. Biol. Chem.*, **248**, 3910 (1973).

(14) K. Wuthrich, *Struct. Bonding (Berlin)*, **8**, 53 (1970).
(15) (a) G. Redfield and R. J. Gupta, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 405 (1971); (b) C. C. McDonald and W. D. Phillips, *Biochemistry*, **12**, 3170 (1973).
(16) (a) R. M. Keller, G. W. Pettigrew, and K. Wuthrich, *FEBS Lett.*, **36**, 151 (1973); (b) C. C. McDonald, W. D. Phillips, and J. LeGall, *Biochemistry*, **13**, 1952 (1974); (c) G. M. Smith and M. D. Kamen, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4303 (1974).
(17) A. Kowalsky, *Biochemistry*, **4**, 2382 (1965).
(18) H. Davenport, *Nature (London)*, **169**, 75 (1952).
(19) F. Sherman, J. W. Stewart, J. H. Parker, E. Inhaber, N. A. Shipman, G. J. Putterman, R. L. Gardisky, and E. Margoliash, *J. Biol. Chem.*, **243**, 5446 (1968).
(20) H. Muir and A. Neuberger, *Biochem. J.*, **45**, 163 (1949).
(21) H. Budzikiewicz, C. Djerassi, and D. Williams, "Mass Sepctrometry of Organic Compounds", Holden-Day, San Francisco, Calif., 1967, pp 628–629.
(22) A. N. H. Yeo, *Chem. Commun.*, 1154 (1970).
(23) T. Popper and H. Tuppy, *Acta Chem. Scand.*, **17**, S47 (1963).
(24) (a) H. Theorell, *Biochem. Z.*, **301**, 201 (1939); (b) D. Theodoropoulos and I. Souchleris, *J. Org. Chem.*, **31**, 4009 (1966); (c) H. Gnichtel and W. Lautsch., *Chem. Ber.*, **98**, 1647 (1965).
(25) (a) H. Theorell, *Biochem. Z.*, **295**, 207 (1936); (b) *ibid.*, **298**, 242 (1938); (c) *ibid.*, **301**, 201 (1939); (d) *Enzymologia*, **4**, 192 (1937).
(26) J. B. Paine and D. Dolphin, *J. Am. Chem. Soc.*, **93**, 4080 (1971).
(27) R. Bonnett, I. A. D. Gale, and G. F. Stephenson, *J. Chem. Soc. C*, 1600 (1966).
(28) S. J. Jacobson, C. G. Willson, and H. Rapoport, *J. Org. Chem.*, **39**, 893 (1974).
(29) G. W. Kenner, S. W. McCombie, and K. M. Smith, *Justus Liebigs Ann. Chem.*, 1362 (1973).
(30) R. P. Carr, A. H. Jackson, G. W. Kenner, and G. S. Sach, *J. Chem. Soc. C*, 487 (1971).
(31) The small but distinct differences between synthetic and natural porphyrin c will be discussed in a future publication.
(32) Solvent evaporations were carried out in vacuo using a Berkeley rotary evaporator, ultraviolet and visible spectra were measured on a Cary Model 14 spectrometer, NMR spectra were taken on Varian Model T-60, HA-100, and HR-220 spectrometers using internal Me_4Si, and mass spectra were obtained on CEC 103 and 110B and A.E.I. MS9 spectrometers. The 200-tube automatic Craig type distribution train was manufactured by H. O. Post Scientific Instrument Co. Radioactivity determinations were made with Packard TriCarb equipment; sample oxidation in sample oxidizer, Model 305, and counting in liquid scintillation counter, Model 3375. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley.
(33) V. du Vigneaud, L. F. Audreith, and H. S. Loring, *J. Am. Chem. Soc.*, **52**, 4500 (1930).
(34) D. Seebach, B. Erickson, and G. Singh, *J. Org. Chem.*, **31**, 4303 (1966); A. Vallet, A. Janin, and R. Romanet, *J. Labelled Compd.*, **4**, 299 (1968).
(35) C. S. Marvel and F. Hager, "Organic Syntheses", Collect. Vol. I, Wiley, New York, N.Y., 1944, p 248.
(36) The preparation of $[2\text{-}^2H_1]$ethanol has been reported via $LiAlD_4$ reduction of ethylene oxide by E. Bengsch and M. Corval, *Bull. Soc. Chim. Fr.*, 1867 (1963). In our hands this procedure resulted in significant quantities of both dideuterio and unlabeled products. The isotopic scramble is difficult to detect by NMR or by mass spectral analysis of the alcohol because pressure-dependent intermolecular proton transfer in the mass spectrometer and the natural isotopic mixture of oxygen renders the M^+ region complex. In the derived iodide, these problems are both obviated and the molecular ion region of the iodide mass spectrum is uncomplicated and accurately reveals this isotopic mixture.
(37) M. M. Kreevoy and M. A. Turner, *J. Org. Chem.*, **30**, 373 (1965).

Lanthanide Interactions with Nitrotyrosine. A Specific Binding Site for Nuclear Magnetic Resonance Shift Probes in Proteins

Timothy D. Marinetti, Grayson H. Snyder, and Brian D. Sykes*

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received March 20, 1975*

Abstract: A 100-MHz ^1H NMR study of the relaxation and chemical shifts induced by the interaction of Eu(III), Pr(III), Gd(III), and La(III) with *N*-acetyl-L-3-nitrotyrosine ethyl ester has been undertaken to characterize the nitrotyrosine residue as a potential specific lanthanide metal binding site in proteins. The NMR data, together with independent measurements of the metal–ligand stability constants, give the parameters of the dipolar interaction. These indicate a significant contribution from nonaxially symmetric terms. In addition, a nonnegligible contact shift is present for Eu(III).

The utility of the lanthanide shift probes as an aid in determining molecular structure and conformation in solution has been well documented over the last several years.[1,2] Most of the work to date has involved the use of "shift reagents" [e.g., $Ln(fod)_3$] in organic solvents. Increasingly more interest is being shown in using the aquo cations themselves, or their EDTA complexes as aqueous shift reagents, for structure determination in aqueous solution. In particular, several molecules of biochemical interest have been studied including mononucleotides,[3,4] dinucleotides,[5] and membranes.[6,7]

Recently the Oxford enzyme group has been investigating the use of the lanthanide binding to a specific site on the protein lysozyme to determine the three-dimensional structure of the protein in solution with minimal reference to the known X-ray structure.[8,9] A limitation of the extension of this technique to other proteins is that the protein must possess a single specific naturally occurring binding site. Although the lanthanides are reported to bind to several proteins such as thermolysin,[10] α-amylases,[11] trypsinogen,[12] and ferredoxin,[13] this will obviously not be true for all proteins of interest. One way to circumvent this difficulty is the incorporation of a binding site into the protein.[9] We have therefore investigated the chelating group nitrotyrosine which can be introduced with relative ease into a large number of proteins.[14] In many of these proteins, nitration occurs at only one tyrosine, hence generating a single binding site for the metal ion.

This paper reports the study of the interaction of Pr(III), Eu(III), and Gd(III) with the model peptide *N*-acetyl-L-3-nitrotyrosine ethyl ester by measurement of the induced NMR shifts, line widths, and spin-lattice relaxation times. In addition, by potentiometric titration, the binding constants of these metals and La(III), a diamagnetic analog of the 4f series, have been determined. With known binding constants, the equilibrium composition of the samples used in NMR can be calculated, and, hence, data involving a change in a spectral parameter as a function of the concentration of paramagnetic metal ion can be analyzed to extract the parameters for the metal–ligand complex. Using the isotropic relaxation probe Gd(III) and the known X-ray structure of nitrophenol, the location of the metal binding

* Address correspondence to this author at Department of Biochemistry, The University of Alberta, Edmonton, Alberta, Canada T6G-2H7.