Stereochemistry of the Porphyrin–Protein Bond of Cytochrome *c*. Stereochemical Comparison of *Rhodospirillum rubrum*, Yeast, and Horse Heart Porphyrins *c*†

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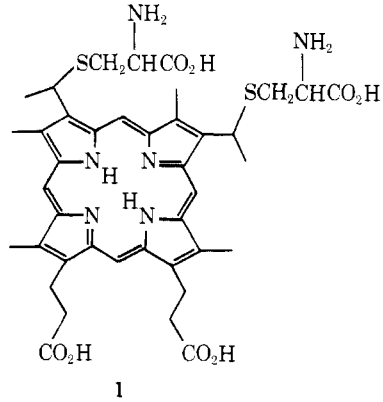
ABSTRACT: Porphyrins *c* have been obtained from *Rhodospirillum rubrum* cytochrome c_2, yeast cytochrome *c*, and horse heart cytochrome *c* and compared using proton magnetic resonance and circular dichroism. Identity of the spectra establishes that chemically and stereochemically the three porphyrins *c* are identical. Since the stereochemistry of the porphyrin α-thioether linkage is not affected in the conversion to porphyrin *c*, the stereochemistry at the porphyrin α-thioether bonds among the corresponding cytochromes *c* also must be the same. Differences between the proton magnetic resonance of *R. rubrum* cytochrome c_2 and horse heart cytochrome *c* which were rationalized by invoking an opposite stereochemistry at these condensation sites (Smith, G. M., and Kamen, M. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 4303) must therefore be attributed to other factors.

The importance of cytochrome *c* in both eukaryotic and prokaryotic metabolism as well as its relatively low molecular weight and easy accessibility have caused this protein to be the subject of numerous studies (Margoliash and Schejter, 1966; Lemberg and Barrett, 1973). Various bacteria have been found to contain *c*-type cytochromes which resemble the mammalian cytochrome in certain respects (Kamen et al., 1971). Cytochrome c_2 of the photosynthetic bacterium *Rhodospirillum rubrum* is one such protein and is thought to function in photosynthetic electron transport (Smith et al., 1973). This material is closely homologous to mammalian mitochondrial cytochrome *c*, as indicated by comparison of electronic absorption spectra (Horio and Kamen, 1961), amino acid sequence (Dus et al., 1968), and folding pattern as revealed by x-ray analysis (Salemme et al., 1973).

Proton magnetic resonance spectroscopy (^{1}H NMR)[1] has assumed increasing importance as a tool for the study of the structure of small proteins in aqueous solutions. Detailed ^{1}H NMR studies have been carried out on horse heart cytochrome *c* (Redfield and Gupta, 1971; McDonald and Phillips, 1973) and horse heart apocytochrome *c* (Cohen et al., 1974) as well as on several microbial cytochromes *c* (Keller et al., 1973; McDonald et al., 1974). In a recent ^{1}H NMR study of the *R. rubrum* protein, Smith and Kamen (1974) have noted that in ferricytochrome c_2 the resonances assigned to the thioether bridge methyls occur at distinctly different chemical shifts than do those assigned to the corresponding groups in the mammalian (horse heart) protein. They conclude that the differences in chemical shifts can be explained as due to different stereochemistry at the heme α-thioether linkages in the two cytochromes. This interpretation is then extended to stereochemistry at the Cys-14 and Cys-17 bonding to the heme as of the *S* configuration for the *R. rubrum* cytochrome, which is opposite to the assumed *R* configuration advanced for the horse heart cytochrome *c* (Redfield and Gupta, 1971).

This prediction of opposite stereochemistries at the heme α-thioether linkages in these two proteins is of considerable interest. Such a phenomenon would raise intriguing questions about the biosynthesis of these two classes of cytochromes and the effect of the thioether stereochemistry on the biological activity of the cytochrome. Further, this prediction appeared amenable to experimental test by isolation of the two α-thioether moieties in some form which permitted a direct stereochemical comparison between those from cytochrome *c* and those from cytochrome c_2. Such conversions and manipulations must be established to be free from any possibility of racemization or asymmetric induction. By this criterion, heme silver salt cleavage to produce an optically active hematohemin and the sodium amalgam cleavage in D_2O leading to a potentially optically active mesoporphyrin are both not suitable, as discussed by Slama et al. (1975), since the mechanisms for these reactions are unknown.

Since porphyrin *c* [2,4-bis(α-*S*-cysteinylethyl)deuteroporphyrin IX] (**1**) contains the α-cysteinyl thioether linkages



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and only two amino acid residues, it is the ideal substance with which to test the predicted stereochemical differences. This substance is readily available from the acidic hydrolysis of cytochrome *c*, and has been obtained in a highly purified form. More importantly, evidence from the same study indicates that neither racemization nor rearrangement occurs during the hydrolysis and isolation (Slama et al., 1975). Also, this structure contains an intense chromophore adjacent to the two chiral centers and porphyrin *c* exhibits circular dichroism (CD)

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[1] Abbreviations used are: ^{1}H NMR, proton magnetic resonance; CD, circular dichroism; UV, ultraviolet; CCD, countercurrent distribution.

associated with its Soret absorption at 406 nm. Therefore, a comparison of the CD spectra of porphyrins *c* from any cytochromes *c* should establish if the stereochemistries at the α-thioether linkages are the same or different.

In the present study, cytochrome *c* from baker's yeast and cytochrome c_2 from *R. rubrum* were isolated and purified using literature procedures. Porphyrins *c* and c_2 were prepared from these compounds and compared to the porphyrin *c* from horse heart cytochrome *c* using circular dichroism and ^1H NMR spectra.

Materials and Methods

General. Ultraviolet and visible spectra were measured on a Cary Model 14 spectrometer, and NMR spectra were taken on a Brucker 360-MHz spectrometer, located at the Stanford Magnetic Resonance Laboratory. Chemical shifts were measured using the residual protium of CF_3CO_2D (δ 11.4) as a reference. Circular dichroism spectra were measured on an instrument assembled to measure CD and magnetic circular dichroism in the Laboratory of Chemical Biodynamics, University of California, Berkeley, and described in detail by Sutherland et al. (1974). The spectrometer was calibrated using a *d*-camphor-10-sulfonic acid standard. Computer interfacing of the instrument allowed signal averaging, and a sufficient number of scans were taken for each sample to obtain a suitable signal-to-noise ratio (usually from 10 to 30 passes per sample). Absorbances of between 0.8 and 1.0 were used, with a 1.0-cm path length. A constant slit width of 1.0 mm affording a spectral bandwidth of less than 4 nm was employed throughout. Scan speed was either 2.5 or 5.0 Å/s with a time constant of 0.3 s. The 200-tube automatic counter current distribution train was manufactured by H.O. Post Scientific Instrument Co. Optical rotations were measured at the sodium D line on a Bendix Ericsson ETL-NPL Automatic Polarimeter, Type 143A. Amino acid analysis was performed using a Beckman Model 120C amino acid analyzer.

The *R. rubrum* culture, strain S-1, obtained from the Laboratory of Chemical Biodynamics, was received originally from R. Stanier, Department of Bacteriology, University of California, Berkeley (Aug 1966). This organism was grown in completely filled glass-stoppered Pyrex bottles with incandescent illumination and at ambient temperature. A complex growth medium was used consisting of 2 g of casamino acids (Bacto, Technical grade, Difco Laboratories, Detroit, Mich.), 3 g of yeast extract (Bacto certified, Difco Laboratories), and 20 mL of 1 M potassium phosphate (pH 6.8) per liter. Potassium phosphate was sterilized separately and added to the media just prior to inoculation. For mass cultures, 5-gal Pyrex carboys were inoculated with a 5–10% inoculum of a 24–48-h-old culture. Carboys were filled, sealed, and illuminated with a 150-W incandescent flood lamp. Agitation was provided with a magnetic stirring assembly, and bottles were cooled by means of a fan. Cultures were harvested after 5 to 7 days and usually yielded 3.4–3.7 g of packed, wet cells per liter of culture.

Horse heart cytochrome c was obtained from Calbiochem, San Diego, Calif. (A grade, equine heart, salt free, assay 88.3%).

Yeast Cytochrome c. Fleischman's pressed baker's yeast (27 kg) was lysed and the cytochrome absorbed on weak cation exchange resin and purified as described (Sherman et al., 1968), except that ammonium sulfate precipitation was omitted and a stepped rather than a linear gradient was employed in the final chromatography. Yields of about 60 mg of cytochrome *c* per kg were obtained.

Cytochrome c_2 from R. rubrum. The isolation and purification of cytochrome c_2 follow the literature procedure (Bartsch, 1971; Bartsch et al., 1971) except that crystallization from ammonium sulfate was omitted. Yields were about 23 mg of cytochrome c_2 per 100 g of wet packed cells.

The primary standard for purity for this heme protein is its electronic absorption spectrum (Horio and Kamen, 1961; Kamen et al., 1963), particularly the ratio $A_{550}^{red.}/A_{280}^{oxid.}$. This index for the best preparations (95% pure) ranges from 1.12 to 1.20. The index for our cytochrome c_2 was typically 1.17. A related standard, $A_{273}^{red.}/A_{415}^{red}$, was described (Bartsch, 1971), and a value close to 0.24 was reported for the best material. Our preparation showed a $A_{273}^{red.}/A_{415}^{red.}$ equal to 0.21. This cytochrome was also examined by gel electrophoresis, both in Tris-HCl buffer at pH 8.9 and in the presence of sodium dodecyl sulfate, and found to be homogeneous in both systems.

Electrophoresis. Polyacrylamide gel electrophoresis was conducted using the Tris-glycine system of Jovin et al. (1964) as modified (Nagel and Schachman, 1975) except that the lower gel consisted of 11.25% acrylamide and 0.2% *N,N'*-methylenebisacrylamide. Electrophoresis was performed at a current of 1 to 2 mA per tube for 1–2 h until the tracking dye, bromophenol blue, had migrated to within 0.5 cm of the tube bottom. Gels were stained for 2–3 h in 9.25% Coomassie brilliant blue R-250 and destained electrochemically.

Sodium dodecyl sulfate gels were run using the system described by Laemmli (1970) with 10% acrylamide and 0.27% *N,N'*-methylenebisacrylamide. Gels were stained with 0.25% Coomassie brilliant blue R-250 and destained by diffusion at 37 °C in a 5% methanol–7.5% acetic acid–water solvent.

Porphyrins c. Horse heart cytochrome *c* was treated to remove the iron and subjected to sulfuric acid hydrolysis; the resulting porphyrin *c* was isolated and purified by 100-transfer countercurrent distribution (Slama et al., 1975). By identical procedures, the porphyrins *c* were obtained from yeast cytochrome *c* and *R. rubrum* cytochrome c_2.

Synthetic Porphyrin c. Synthetic porphyrin *c* was prepared by modifications of the Sano procedure (Sano et al., 1964), especially in the isolation and purification. Hematoporphyrin dihydrochloride (1.00 g, 1.49 mmol), dissolved in 750 mL of 0.2 N KOH, was heated to 80 °C with mechanical stirring and maintained under a slight nitrogen pressure in the dark while 200 g of 2% sodium amalgam was added. After 15 min, the clear, colorless solution was filtered through sintered glass in the dark and under N_2 to remove mercury. The filtrate was treated with 3 g (17.6 mmol) of L-cysteine hydrochloride in 100 mL of deaerated water and immediately diluted to 3.5 L with glacial acetic acid; the resulting clear solution was set aside overnight, open to the air but in the dark. The dark red resulting solution was diluted to 6 L, adjusted to pH 3.5 with sodium hydroxide, and then passed through a column of Biorex 70 (H^+ form). The column was washed with 2 L of 0.02 M pyridine–acetic acid buffer (pH 5) and then with 4 L of water, and finally the porphyrin was eluted as a sharp band with 30% (v/v) pyridine/water. The bright red eluent was lyophilized to leave 791 mg of crude porphyrin which was taken into water, precipitated at pH 4, collected on a Teflon Millipore filter, and dried at room temperature and under high vacuum over P_2O_5 to leave 768 mg of crude porphyrin *c*.

The crude porphyrin was subjected to a 100-tube countercurrent distribution between pH 2 citrate buffer and 1-butanol to give a brown fraction of high K_D and the product at K_D = 0.4. The product peak was collected, the porphyrin was driven into the aqueous layer by addition of cyclohexane, and the aqueous phase was adjusted to pH 3.5 and passed through

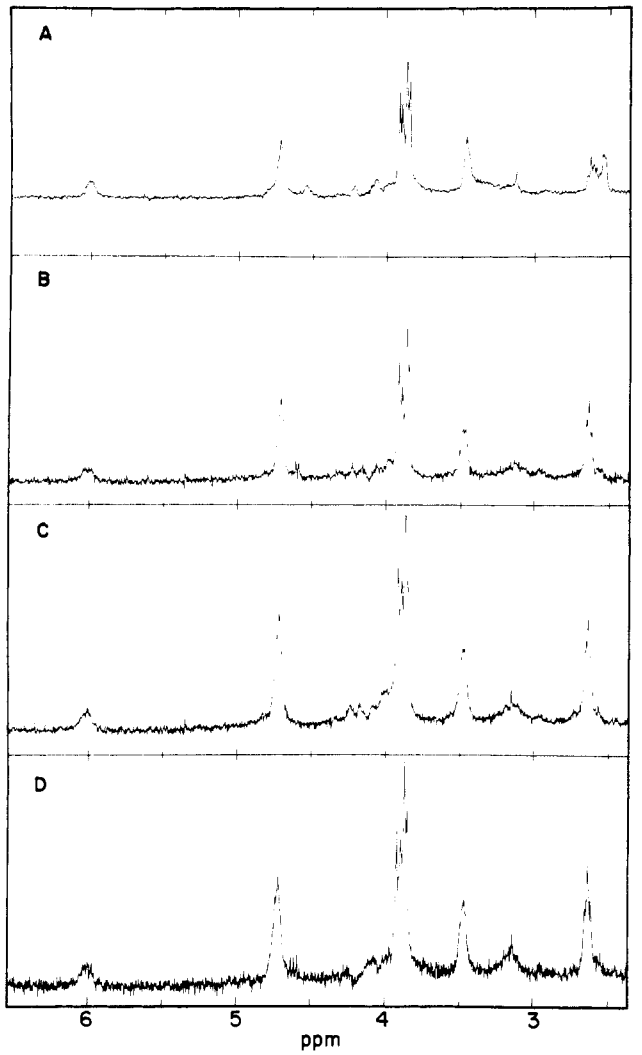


FIGURE 1: ^{1}H NMR spectra at 360 MHz in CF_3CO_2D of: (A) synthetic porphyrin *c*; (B) horse heart porphyrin *c*; (C) *R. rubrum* c_2 porphyrin *c*; and (D) yeast porphyrin *c*.

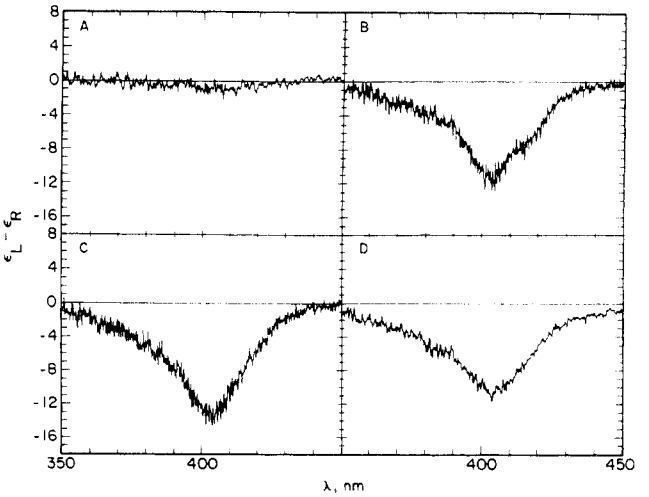


FIGURE 2: Circular dichroism spectra in 20% pyridine/water (v/v) of: (A) synthetic porphyrin *c*; (B) horse heart porphyrin *c*; (C) *R. rubrum* c_2 porphyrin *c*; and (D) yeast porphyrin *c* at room temperature and a concentration of 5.7×10^{-6} M.

Biorex 70. After washing the column with 2 L of water, the porphyrin was eluted with 30% pyridine/water, lyophilized, and precipitated as before to leave 269 mg of porphyrin *c*.

The resulting sample was subjected to a second 100-tube countercurrent distribution and isolated in the same fashion to give 194 mg of synthetic porphyrin *c* as a hygroscopic amorphous powder: UV [1 N HCl, λ_{max} nm ($\epsilon \times 10^{-4}$)] 406 (34.2), 552 (1.56), 594 (0.57). A portion was dried for 18 h at 100 °C (10 μm) for analysis.

Anal. Calcd for $C_{40}H_{48}N_6S_2O_8$: C, 59.7; H, 6.0; N, 10.4. Found: C, 59.4; H, 6.2; N, 10.0.

Optical Stability of L-Cysteine and S-Benzyl-L-cysteine under Conditions of Porphyrin c Synthesis. a. L-Cysteine. To 75 mL of 0.02 M potassium hydroxide at 80 °C under a N_2 blanket was added 20 g of 2% (w/w) sodium amalgam with vigorous stirring. After 15 min, the solution was filtered through a sintered glass filter and a deoxygenated solution of L-cysteine hydrochloride monohydrate (344 mg, 1.75 mmol) in 10 mL of water was added to the filtrate. After 30 s of thorough mixing, 275 mL of glacial acetic acid was added and the resulting mixture was allowed to stand overnight in the dark exposed to the atmosphere. After 15 h, the solution was evaporated to dryness in vacuo, the residue was dissolved in 15 mL of water and lyophilized, and the resulting powder was dissolved in 3 mL of water, cooled to 0 °C, and treated with 1 mL of concentrated HCl. Bromine (0.9 g, 5.8 mmol) was then added and, after 90 min of stirring, the solution was evaporated in vacuo and the residue dissolved in 5 mL of water and lyophilized. The resulting cysteic acid and salt mixture, in water, was adsorbed on a column of Dowex-2 (2 cm × 10 cm, Cl^- form, 50–100 mesh) which was washed with 500 mL of water and the amino acid eluted with 1 M HCl. Lyophilization left 270 mg of cysteic acid, $[\alpha]_D$ +11.1° (*c* 1.9, 1 M HCl). An authentic sample of L-cysteic acid had $[\alpha]_D$ +9.8° (*c* 0.97, 1 M HCl).

b. *S*-Benzyl-L-cysteine. A sample of *S*-benzyl-L-cysteine was exposed to the same conditions as described above through the first lyophilization step. The *S*-benzylcysteine was recovered in 78% yield by crystallization from water. It had $[\alpha]_D$ −14.0° (*c* 1, 5 M HCl), and the starting *S*-benzyl-L-cysteine had $[\alpha]_D$ −14.6° (*c* 1, 5 M HCl).

Results and Discussion

The porphyrins *c* were prepared from horse heart, yeast, and *R. rubrum* cytochromes *c* by identical procedures and purified by countercurrent distribution (CCD). In addition to the characteristic and identical behavior exhibited in the CCD, each compound was characterized by electronic absorption spectra and solubility, fully in agreement with those reported for porphyrin *c*. A 360-MHz ^{1}H NMR spectrum of each of these compounds was obtained, and the spectra were found to be identical as seen in Figure 1. These spectra show the sharp absorption at δ 2.63 characteristic of the α-thioether bridge methyl group, as well as the signal at δ 6.0 assigned to the thioether methine (Slama et al., 1975).

The circular dichroism spectra of the horse heart, yeast, and *R. rubrum* porphyrins *c* have been examined using pyridine/water and 1 N HCl (i.e., porphyrin dication) as solvents over the spectral region associated with the Soret band, from 350 to 450 nm. In pyridine/water, Figure 2, these spectra show a broad negative absorption peaking at 403 nm ($\Delta\epsilon \simeq -11$), while in 1 N HCl, Figure 3, two absorptions are observed, one at 405 nm ($\Delta\epsilon \simeq -9$) and a second at 414 nm ($\Delta\epsilon \simeq -8$). Examination of the spectra of the three compounds reveals identity within experimental error.

In order to interpret these data, it is necessary to keep in

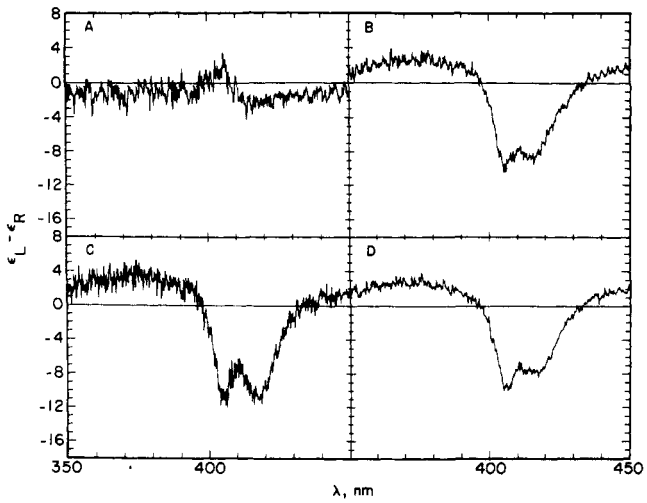


FIGURE 3: Circular dichroism spectra in 1 M HCl of: (A) synthetic porphyrin *c*; (B) horse heart porphyrin *c*; (C) *R. rubrum* c_2 porphyrin *c*; and (D) yeast porphyrin *c* at room temperature and a concentration of 2.5×10^{-6} M.

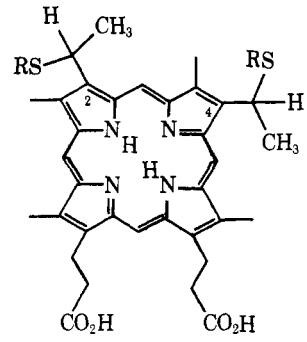
mind that the structure of porphyrin *c* permits four asymmetric centers to exist—the two α-thioether carbons and the two L-cysteinyl α carbons. Although it is entirely reasonable to expect that the L-cysteinyl chiral centers, removed as they are by four atoms from the porphyrin chromaphore, will not contribute to the observed CD spectra, it is possible to evaluate the extent of this interaction using a particular preparation of synthetic porphyrin *c*.

The synthetic porphyrin *c* which results from the addition of L-cysteine to proto- or hematoporphyrinogen (Sano et al., 1964) would be expected to exist as a mixture of configurations about the α-thioether carbons. On the other hand, the optically active L-cysteine center four atoms away from the porphyrin chromophore is retained unchanged since the conditions for formation of the thioether bonds do not cause racemization or inversion of the amino acid chirality, as we have demonstrated (see Materials and Methods).

A porphyrin-cysteine adduct prepared in this way by the addition of L-cysteine to hematoporphyrinogen and purified by CCD shows an NMR spectrum, Figure 1, similar to that obtained from the natural porphyrins *c*. A significant difference is observed in the α-thioether methyl region, however, where the synthetic porphyrin *c* show signals at δ 2.63 and 2.54 as well as considerable broadening. These differences are clearly attributable to the existence of a complex mixture of compounds differing only in their configuration about the α-thioether bridge carbon in the case of synthetic porphyrin *c*.

Since this synthetic porphyrin *c* contains the optically active L-amino acid and a mixture of configurations about the α-thioether carbon, the CD spectrum of such a preparation should reveal any interaction between the amino acid chiral center and the porphyrin chromophore. However, the circular dichroism spectra of the synthetic porphyrin *c* in pyridine/water and in 1 N HCl, Figures 2 and 3, show essentially no activity. The absence of any signal for this compound simultaneously eliminates the possibility of any asymmetric induction in the thioether bond forming step of the synthesis and also establishes the absence of any contribution from the amino acid chiral center to the circular dichroism.

For the purpose of interpreting the porphyrin *c* CD spectra, it is thus possible to ignore the presence of the chiral amino acid. Considering only the two α-thioether carbons, it is apparent that four isomeric porphyrins must exist, **2a,b,c,d,** related as two pairs (**a,b** and **c,d**) of enantiomers. Starting with



2a, C-2, *R*; C-4, *R*
b, C-2, *S*; C-4, *S*
c, C-2, *R*; C-4, *S*
d, C-2, *S*; C-4, *R*

any one of these four compounds, if we invert both of the chiral centers (that is, give each chiral center the opposite configuration, as was predicted for *R. rubrum* c_2 porphyrin *c* compared to horse heart porphyrin *c*), then an enantiomer will be the result whose CD spectrum must be the mirror image of that of the starting compound. If only one of the chiral centers is inverted, the substance is converted to a diastereomer and should also have a different CD spectrum. Hence, if two porphyrins *c* have identical CD spectra, then it must reflect identical stereochemistries about the α-thioether linkages.

The above analysis leads to the conclusion that the porphyrins *c* derived from horse heart, yeast, and *R. rubrum* cytochromes are identical, both in structure and in stereochemistry about the α-thioether linkage. This requires that the configuration of these cytochromes about the α-thioether bond must also be the same. Differences observed in the ^{1}H NMR spectra of *R. rubrum* cytochrome c_2 and horse heart cytochrome *c* must therefore be due to some other, as yet undefined, factors.

The fact that the thioether bonds are formed with the same stereochemistry in three distantly related organisms, horse, yeast, and *R. rubrum*, may be coincidence or may reflect similarities in the mechanism of biosynthesis of the cytochromes *c*. This stereochemistry may also have a role in the biological function of the cytochromes *c*.

Acknowledgment

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In Vitro Reconstitution of Calf Brain Microtubules: Effects of Solution Variables†

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ABSTRACT: The effects of solution variables on the in vitro reconstitution of calf brain tubulin, purified by the method of Weisenberg et al. (Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7,* 4466–4479; Weisenberg, R. C., and Timasheff, S. N. (1970), *Biochemistry 9,* 4110–4116), as modified by Lee et al. (Lee, J. C., Frigon, R. P., and Timasheff, S. N. (1973), *J. Biol. Chem. 248,* 7253–7262), were investigated at pH 7.0. Reconstitution of microtubules was successful in a variety of buffer systems, the free energy of the propagation step of microtubule formation being little dependent on the buffer. Microtubule formation is promoted by magnesium ions and guanosine triphosphate, but inhibited by calcium ions. The dependence of the apparent association constant for microtubule formation on ligand concentration was analyzed by the linked function theory of Wyman (Wyman, J. (1964), *Adv. Protein Chem. 19,* 224–286), leading to the conclusion that the formation of a tubulin–tubulin contact involves the binding of one additional magnesium ion per tubulin dimer. Microtubule formation is also accompanied by the apparent binding of one additional proton and the release of water molecules, as suggested by the thermodynamic parameters determined. The reaction is entropy driven with an apparent heat capacity change, ΔC_p, of -1500 ± 500 cal/deg-mol. The enhancement of tubulin reassembly by glycerol is most likely due to nonspecific protein–solvent general thermodynamic interactions.

Following the initial observations of Weisenberg (1972) that microtubules can be reconstituted from a partially purified tubulin preparation, a number of reports have appeared on the in vitro reconstitution of microtubules (Olmsted and Borisy, 1975; Jacobs et al., 1974, 1975; Shelanski et al., 1973; Kirschner et al., 1974; Erickson, 1974). In all of these studies, the protein was prepared according to several variants of the polymerization–depolymerization (cycle) procedure of Shelanski et al. (1973). This procedure yields tubulin of 80 to 90% purity, the rest consisting of 200 000 to 300 000 molecular-weight components and/or smaller components distributed into 10 to 15 different protein bands observed in sodium dodecyl sulfate gel electrophoresis (Weingarten et al., 1975; Erickson, 1974; Murphy and Borisy, 1975). Results obtained with tubulin prepared by the cycle procedure have led Kirschner and co-workers (Kirschner et al., 1974; Kirschner and Williams, 1974) to conclude that the 5.8S native dimers of tubulin (110 000 molecular weight) are chemically and functionally different from the subunits which make up ring structures, that the ring structures are not in equilibrium with the 5.8S species, and that only rings polymerize into microtubules, the 5.8S species being unable to do so. Furthermore, these authors have asserted that the ability of the rings to assemble into microtubules is due to a salt-dissociable factor. In the absence of such a factor, they have stated that "tubulin is utterly unable to polymerize into microtubules" (Weingarten et al., 1975). These conclusions are in contrast with the results obtained with

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