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Porphyrin–Protein Bond of Cytochrome c_{558} from *Euglena gracilis*

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Abstract: The atypical cytochrome c_{558} isolated from dark-grown cultures of *Euglena gracilis* contains a single heme to protein thioether bond. Sodium amalgam reductive cleavage of this bond followed by esterification yielded a porphyrin which is identical with synthetic 2-vinyl-4-ethyldeuteroporphyrin IX dimethyl ester and different from its 2-ethyl-4-vinyl isomer. This establishes *Euglena* porphyrin c_{558} as 2-vinyl-4-(α-S-cysteinylethyl)deuteroporphyrin IX, and the monothioether linkage in cytochrome c_{558} is at the 4-α-ethyl position of the heme. Implications of this result on the sense of the porphyrin ring relative to the peptide chain in cytochromes c with two thioether linkages are considered.

Cytochromes c are proteins which contain a covalently bound iron–porphyrin prosthetic group and are active in the electron-transport processes in photosynthesis and respiration.[1] While considerable evidence had suggested that the covalent linkage is through two thioether bonds involving two cysteine residues of the apoprotein, it was not until recently that studies with yeast and horse heart cytochrome c firmly established porphyrin c, the porphyrinic substance obtained from acid hydrolysis of cytochrome c, as 2,4-di(α-S-cysteinylethyl)deuteroporphyrin IX.[2] This also established the α-thioether linkages in cytochrome c. Consistent with the generality of two thioether linkages to the heme group has been

the finding that in the more than 60 cytochromes c for which amino acid sequences are known, all contained the partial sequence ···Cys·X·Y·Cys·His···.[3,4]

Thus the two cysteine residues were considered invariant and hence essential until the recent demonstration that in *Crithidia oncopelti* cytochrome c_{557}[5] and *Euglena gracilis* mitochondrial cytochrome c_{558}[6] the heme group is covalently bound through only one thioether linkage; yet the cytochromes are functional. Therefore, it is of considerable interest to determine the exact nature of this monothiother bond since it must now be considered the minimum linkage necessary for the function of at least some of the c-type cytochromes. De-

termination of the position of the linkage, that is, at C-2 (**1a**) or C-4 (**1b**) of the porphyrin ring, should also reflect on the general question of the sense of the porphyrin ring relative to the peptide chain, assuming that it is the same in other cytochromes c.

In order to completely elucidate the nature of the single heme to apoprotein linkage in these atypical cytochromes c_{557} or c_{558}, it was desirable to establish (a) that the linkage truly is a thioether bond, (b) why there is only one such bond, (c) at what position the linkage is in the peptide chain, (d) the nature of the "unbound" position on the heme, and (e) which of the two possible positions of attachment to the heme is utilized. Recent reports in the literature have established all but the last of these points.[4-8]

While cytochrome c_{552} obtained from *E. gracilis* grown in the presence of light contains the usual sequence pattern ···Cys[10]·X·Y·Cys[13]·His···, and, consequently, has the normal dithioether linkage to the heme,[9] the cytochrome c_{558} obtained from the same strain contains instead the sequence ···Ala[14]·X·Y·Cys[17]·His···.[8] Thus the cysteine at position 14 needed to form the second thioether bond is replaced by alanine. The bathochromic shift to 558 nm in the visible spectrum of the latter cytochrome suggested that the unbound position of the heme remained as a free vinyl group, and a positive test for a free vinyl group was consistent with this suggestion.[8]

No evidence has been presented on whether the thioether linkage is at the 2 position (**1a**) or at the 4 position (**1b**) of the heme or, less likely, whether the natural cytochrome contains a mixture of the two isomeric hemes. We wish to report the unambiguous determination of *E. gracilis* porphyrin c_{558} as 2-vinyl-4-(α-S-cysteinylethyl)deuteroporphyrin IX (**1b**), thus establishing that the monothioether linkage of cytochrome c_{558} is at the 4-α-ethyl position of the heme prosthetic group.

Euglena gracilis klebs variety *bacillaris* was cultivated in the dark, the cells were harvested and lysed, the debris was removed by centrifugation, and the cytochrome c_{558} (isoelectric point 9.55) was adsorbed onto a sodium carboxylate cation-exchange resin, washed, eluted, and finally purified in both the reduced and oxidized form by chromatography on a cation-exchange resin.[4-8] Removal of the porphyrin prosthetic group from the apoprotein in a manner that retained the natural distinction of the 2 and 4 positions was then necessary. Previous work[10,11] indicated that not only does sodium amalgam reductive cleavage of cytochromes c containing the usual dithioether linkage yield mesoporphyrin **2**, but free vinyl substituents on the periphery of other porphyrins at least partially survive this reductive treatment. In addition, our recent work[2] clearly demonstrated that the sodium amalgam degradation of α-linked cytochromes c proceeds with integrity and the products reflect the structure of the thioether linkage in the original cytochrome c.

Thus, if successful, sodium amalgam reductive cleavage of the thioether bond of *Euglena* cytochrome c_{558} and subsequent recovery of a monovinylmonoethyldeuteroporphyrin would not only establish the thioether bond as α to the porphyrin ring and confirm the presence of a free vinyl group, but identification of which of the two isomers, **3a** or **3b**, was obtained also would specify the position (2- or 4-α-ethyl) of the heme-to-protein linkage. Therefore, it was necessary to be able to distinguish the 2-vinyl-4-ethyl- and 2-ethyl-4-vinyldeuteroporphyrins. Alternatively, the monovinylmonoethyldeuteroporphyrin obtained from the cytochrome could be converted to the simpler monoethyldeuteroporphyrin by reaction in a resorcinol melt, which is known to remove peripheral vinyl substituents from porphyrins.[12] Structural assignment would then require the ability to distinguish 2-ethyldeuteroporphyrin (**4a**) and 4-ethyldeuteroporphyrin (**4b**). With these alternatives in mind, a method of distinguishing 2- and 4-substituted porphyrin isomers was sought.

Synthesis of Porphyrin Isomers. The monoformylation and acetylation of the copper complex of deuteroporphyrin dimethyl ester and the chromatographically separated 2- and 4-isomers have been reported.[13] In the formyl series, the higher melting compound (mp 266–267 °C) was designated 4-formyldeuteroporphyrin dimethyl ester (**5b**) because a compound of similar melting point had been used during the synthesis of *Spirographis* porphyrin, the structure of which has been verified.[14,15] The isomeric methoxycarbonyl derivatives **6a** and **6b** were prepared by oxidation of the formyl groups and subsequent methylation. While the melting points of **6a** and **6b** were essentially identical (214–216 and 213–215 °C), in the NMR the signal assigned to the C-2 proton in both the formyl and methoxycarbonyl series was 5–12 Hz upfield relative to that of the C-4 proton. By analogy, the acetyl isomer with mp 212 °C and the relatively downfield proton was designated 2-acetyldeuteroporphyrin (**7a**) while the isomer which melted at 240 °C and exhibited a relatively upfield proton was assigned the 4-acetyldeuteroporphyrin (**7b**).[13]

However, the recently reported[16] unambiguous total synthesis of both the 2- and 4-acetyldeuteroporphyrin dimethyl esters showed that the previous designations were erroneous; that is, the 2-isomer **7a** melts at 240 °C while the 4-isomer **7b** melts at 212 °C. Moreover, the acetyl derivatives were converted to the corresponding 2-vinyldeuteroporphyrin (**8a**, isopemptoporphyrin) and 4-vinyldeuteroporphyrin (**8b**, pemptoporphyrin), both of which have been previously synthesized and characterized since the latter is a natural product.[14,15,17] The isomeric vinyl isomers were then converted to the formyldeuteroporphyrins **5a** and **5b** by treatment with osmium tetraoxide and oxidation of the resulting diols with sodium periodate. Again comparison with the formyl isomers obtained by direct formylation indicated that the initial assignment[13] had been incorrect.
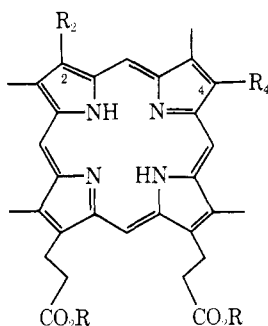
Since the structural assignment of the acylated deuteroporphyrin isomers was now clearly defined, the unambiguous synthesis of the sets of isomeric porphyrins needed to establish the structure of the porphyrin expected from *Euglena* cytochrome c_{558} was initiated. Commerical hemin chloride (**9**) was subjected to the resorcinol melt procedure, the iron was removed with hydrazine, and the product was esterified to give deuteroporphyrin dimethyl ester (**10**) in 39% overall yield. Since Friedel–Crafts type reactions of porphyrins are best performed on the metal chelates,[13] **10** was treated with cupric acetate to give the copper complex in 99% yield. Acetylation by 30-s exposure to stannic chloride and acetic anhydride followed by quenching with water, removal of the copper, and reesterification gave a mixture which was chromatographically separated into three components: deuteroporphyrin dimethyl ester (**10**), 22.5%, recovered starting material; a mixture of 2- and 4-monoacetyldeuteroporphyrin dimethyl ester (**7a** and **7b**), 53%; and 2,4-diacetyldeuteroporphyrin dimethyl ester (**11**), 12%. The desired monoacetyl isomers were separated by fractional crystallization from acetone followed by chromatography of the enriched fractions. The separation and characterization of the monoacetyldeuteroporphyrin dimethyl esters **7a** and **7b** thus provided the required distinction between the 2 and 4 positions on the periphery of the porphyrin ring.

2- and 4-Acetyldeuteroporphyrin were then individually reduced with sodium borohydride to the α-hydroxyethyl isomers **12a** and **12b** in 93 and 92% respective yields. Dehydration with p-toluenesulfonic acid provided 2- and 4-vinyldeuteroporphyrin dimethyl esters (**8a** and **8b**, 89 and 90%) with melting points identical with those reported for the dimethyl esters of isopempto- and pemptoporphyrins,[14-17] therefore reconfirming the structural assignments. Subsequent catalytic reduction of the vinyl groups provided 2- and 4-ethyldeuteroporphyrin dimethyl ester (**4a** and **4b**) in 89 and 85% yields, respectively. Since the melting points (214–215.5 and

213.5–215.5 °C), electronic spectra, and NMR spectra of the monoethyl isomers did not readily distinguish the two separate isomers, the conversion of the porphyrin obtained from *Euglena* cytochrome c_{558} to the monoethyl derivative was not considered productive. Such a conversion would also have required the subjection of scarce material obtained from the natural product to several vigorous and low-yield reactions. Therefore, the separate synthetic monoethyl isomers **4a** and **4b** were carried on to the corresponding desired monovinyl-monoethyl derivatives **3a** and **3b**.

Thus the copper complexes of **4a** and **4b** were prepared and acetylated. Removal of the copper and reesterification gave 2-ethyl-4-acetyl- and 2-acetyl-4-ethyldeuteroporphyrin dimethyl esters (**13a** and **13b**) in 53 and 52% yields, respectively. Again, sodium borohydride reduction afforded the corresponding monohydroxyethylmonoethyl derivatives **14a** and **14b** (90 and 94%) which were dehydrated to the desired 2-ethyl-4-vinyl- and 2-vinyl-4-ethyldeuteroporphyrin dimethyl esters **3a** and **3b**. These isomers could be distinguished by NMR, melting point, and crystal form as shown below. However, an artificial mixture of the final set of isomers could not be separated by high-pressure liquid chromatography although a number of conditions were tried, including those which were reported to be successful for the separation of other porphyrin isomers.[18]

Cleavage of the Porphyrin–Protein Linkage. To maximize conversion of the scarce *Euglena* cytochrome c_{558}, it was de-

	R	R₂	R₄	Mp, °C
1a	H	CHSCH₂CHCO– CH₃ NH–	CH=CH₂	
1b	H	CH=CH₂	CHSCH₂CHCO– CH₃ NH–	
2	CH₃	CH₂CH₃	CH₂CH₃	216
3a	CH₃	CH₂CH₃	CH=CH₂	224–226
3b	CH₃	CH=CH₂	CH₂CH₃	232–234
4a	CH₃	CH₂CH₃	H	214–215.5
4b	CH₃	H	CH₂CH₃	213.5–215.5
5a	CH₃	CHO	H	266–268[16]
5b	CH₃	H	CHO	241–242[16]
6a	CH₃	CO₂CH₃	H	214–216[16]
6b	CH₃	H	CO₂CH₃	213–215[16]
7a	CH₃	COCH₃	H	240[17]
7b	CH₃	H	COCH₃	212[17]
8a	CH₃	CH=CH₂	H	219–221[16]
8b	CH₃	H	CH=CH₂	208–210[14,15,17]
9	H	CH=CH₂	CH=CH₂	(Fe complex)
10	CH₃	H	H	221–223
11	CH₃	COCH₃	COCH₃	240[13]
12a	CH₃	CHOHCH₃	H	236.5–239[13]
12b	CH₃	H	CHOHCH₃	217–221
13a	CH₃	CH₂CH₃	COCH₃	242.5–244
13b	CH₃	COCH₃	CH₂CH₃	256–258
14a	CH₃	CH₂CH₃	CHOHCH₃	208–210
14b	CH₃	CHOHCH₃	CH₂CH₃	201–203
15	H	CH=CH₂	CH=CH₂	

sirable to first determine optimum conditions for the reductive sodium amalgam cleavage, subsequent reoxidation of the porphyrinogen, and final esterification and purification of the resulting porphyrin without loss of distinction between the 2 and 4 positions, that is, cleavage of the thioether linkage without reduction of the free vinyl group to give a mono-vinylmonoethyldeuteroporphyrin, **3a** or **3b**. While mesoporphyrin has been reported in 45–55% spectroscopic yield from cytochrome c via the sodium amalgam reduction followed by air oxidation,[10] no indication of the purity of the material so obtained was given nor was the product subjected to esterification and purification. In our previous work, mesoporphyrin dimethyl ester (**2**) was obtained in 30% isolated yield from horse heart cytochrome c.[2] That the vinyl groups of protoporphyrin (**15**) survived this treatment with sodium amalgam was stated,[10] without stipulating the percent recovery after reoxidation of the resulting porphyrinogen. More recently, a 65% recovery of protoporphyrin was reported[11] along with 15% monovinylmonoethyldeuteroporphyrin and 5–10% mesoporphyrin from the reduction of **15** with Na–Hg at 80 °C for 1.5–2 min followed by air oxidation in the presence of weak light, with no mention being made of how the composition of the product mixture was determined. Spectroscopic analysis of the composition of such a crude reoxidation product may be deceiving, as shown below. Therefore, a series of trials was in order. Sodium amalgam (3%) was prepared and commercial horse heart cytochrome c and protohemin chloride were chosen for study of the total process: Na–Hg reduction, reoxidation, and esterification.

Variations of the reduction temperature (20–80 °C) and time (2–5 min) and length of air reoxidation (3–8 h) were examined, yielding results of interest. Most notable was the observation that crude yields obtained spectroscopically immediately after the air reoxidation (30–60% as reported[10]) were considerably higher than isolated yields (8–32%) of esterified and purified material. In all cases the crude material showed an extra absorbance at 640–660 nm which was inconsistent with the presence of only the desired porphyrin. After esterification, the impurity could be separated from the desired porphyrin chromatographically and usually amounted to a significant fraction, as indicated by the lowered yield of pure desired porphyrin.

We also observed that the yield of the pure desired porphyrin varied considerably with reaction temperature and time. The yield of protoporphyrin from hemin decreases as the reaction temperature and time of exposure to Na–Hg increased, while the opposite was observed for the yield of mesoporphyrin from horse heart cytochrome c. In addition, the yields of protoporphyrin from hemin were considerably lower than the yields of mesoporphyrin from horse heart cytochrome c.

Despite reports in the literature,[19] reaction of the free vinyl groups and a resultant lowering of the yield of protoporphyrin during the sulfuric acid–methanol or diazomethane esterification was suspected. When authentic purified protoporphyrin dimethyl ester was subjected to the sulfuric acid–methanol esterification procedure, it could be recovered in 88% yield. Similarly, protoporphyrin free acid[20] was esterified and purified in an identical manner to give the diester in 78% yield. A sample of synthetic 2-vinyl-4-ethyldeuteroporphyrin dimethyl ester (**3b**) was also subjected to the sulfuric acid–methanol esterification. Isolation revealed no loss of material or any trace of other product as determined by TLC and electronic spectrum. Protoporphyrin dimethyl ester could also be recovered in 88% recrystallized yield after treatment with excess ethereal diazomethane. However, similar treatment of a solution of protoporphyrin free acid with excess ethereal diazomethane in a solution containing the acetic acid necessary to dissolve the porphyrin resulted in only a trace of the desired dimethyl ester. An electronic spectrum of the product mixture

also had a band at 650 nm just as observed in the products of the Na–Hg reductions. From these observations, it was concluded that the methanol–sulfuric acid esterification procedure was the method of choice.

The combined optimum conditions found for the recovery of mesoporphyrin dimethyl ester (**2**) from horse heart cytochrome *c* (32% yield) and protoporphyrin dimethyl ester from hemin **9** (17%) involved treatment with Na–Hg for 1.75 min at 80 °C, extraction, air oxidation of the resulting porphyrinogen for ∼5 h, and methanol–sulfuric acid esterification. Since the porphyrin from *Euglana* cytochrome c_{558} was anticipated to have properties of both horse heart cytochrome *c* (thioether linkage) and hemin (free vinyl group), the outcome of its overall reaction was difficult to predict. In fact, when 2 μmol of the *Euglena* cytochrome was subjected to the reaction conditions, only a 10% yield of desired porphyrin was obtained after extraction, oxidation, esterification, and chromatography. As usual, the chromatography separated the desired porphyrin from a significant amount of a more polar porphyrinic substance which contained an additional absorbance at 650 nm. Comparison of the visible spectrum with those of the synthetic monovinylmonoethylporphyrin isomers revealed that while the porphyrin obtained from *Euglena* was similar to the synthetic isomers, slight differences could be detected. In addition to a trace absorbance at 650 nm the most notable difference was the ratio of the absorbance at 538–540 nm to that at 503 nm, 0.9 for both synthetic isomers **3a** and **3b** but 0.8 for the *Euglena* porphyrin. Further studies of the conversion process were needed, and these focused on the reoxidation step.

Reoxidation of the cleaved porphyrinogen to the desired porphyrin is usually performed by simply exposing the crude acidic extract to air. The oxidation of both porphyrinogens[21] and chlorins[22] to porphyrins with dichlorodicyanobenzoquinone (DDQ) also has been reported; however, sodium amalgam reduction of protohemin **9** followed by DDQ oxidation and esterification yielded no protoporphyrin dimethyl ester. The same conditions on horse heart cytochrome *c* gave no mesoporphyrin. In both experiments the addition of 200–400 mol % of DDQ to the extracts from the sodium amalgam reaction gave an immediate red color, presumably from the conversion of porphyrinogen to porphyrin; yet after complete isolation, including esterification, no desired porphyrin could be obtained.

Since excess DDQ had been used, overoxidation was considered a probable cause. The use of exactly 100 mol % of DDQ was not practical since the efficiency of the sodium amalgam reduction cannot be ascertained and therefore no stoichiometric basis is available. Rapid removal of the excess DDQ after the oxidation by filtering through alumina as suggested[21,22] was also considered impractical because the free porphyrinogen acids could not be eluted from the alumina. Therefore, a mild reduction step was incorporated shortly after the DDQ oxidation to destroy excess oxidant. The yield of desired porphyrin varied considerably with the time allowed before quenching excess DDQ with dithionite,[23] but use of this method—DDQ oxidation of porphyrinogen to porphyrin followed by destruction of the oxidant after 1 min—gave a 37% yield of pure protoporphyrin from hemin and a 53% yield of pure mesoporphyrin from horse heart cytochrome *c* after esterification and chromatography, considerable improvements in both instances.

When *Euglena* cytochrome c_{558} was subjected to the modified overall reductive cleavage conditions, the yield of porphyrin obtained after esterification and chromatography on alumina was improved to 22%. The visible spectrum still showed the presence of a long wavelength absorbing impurity and the same ratio of $A_{538}/A_{502.5} = 0.8$ instead of 0.9 as for the synthetic isomers. The material was then subjected to high-pressure liquid chromatographic (LC) analysis which

revealed two major components identified as a monovinyl-monoethyl derivative and mesoporphyrin by comparison with known compounds and an artificial mixture of the two. The results of the LC analysis indicated that 30% of the recovered *Euglena* (c_{558}) porphyrin had been overreduced to mesoporphyrin. A visible spectrum of an artificial mixture of monovinylmonoethyldeuteroporphyrin dimethyl ester and mesoporphyrin dimethyl ester in the same ratio as determined in the natural material was identical with that of the natural material.

At this point about 1.1 μmol of the porphyrin dimethyl esters had been obtained from *Euglena* as estimated from the absorbance at 503 nm, assuming $\epsilon = 14\,000$ (the same as meso- and protoporphyrin dimethyl esters). After chromatography of the mixture on a thoroughly washed TLC grade silica gel column using nitrogen pressure, a 0.24-mg, 0.41 μmol, fraction of monoethylmonovinyldeuteroporphyrin dimethyl ester [*Euglena* (c_{558}) porphyrin] was obtained, containing a maximum of 2% mesoporphyrin. The visible spectrum of the purified material was identical with that of the synthetic isomers **3a** and **3b** in all respects.

As already indicated LC did not distinguish between the monoethylmonovinyl isomers **3a** and **3b**. Therefore, assignment of the porphyrin obtained from *Euglena* cytochrome c_{558} as either structure **3a** or **3b** depended on the NMR and melting point data. The 360-MHz Fourier transform NMR clearly distinguished the two synthetic isomers, particularly by the pattern of the meso proton and methyl signals. Comparison of the spectra of the synthetic isomers with the spectrum of the *Euglena* (c_{558}) porphyrin (Figures 1 and 2) provided its unambiguous assignment as 2-vinyl-4-ethyldeuteroporphyrin dimethyl ester (**3b**). Since the NMR spectra of porphyrins are known to be concentration dependent,[24] all spectra were recorded at the same concentration, 1 mM, 0.4 μmol/0.4 mL.

Recrystallization of *Euglena* (c_{558}) porphyrin dimethyl ester provided microscopic needles with micro-mp 231–233 °C, thus reconfirming its structure as **3b** since synthetic **3b** also recrystallized as microneedles with micro-mp 232–234 °C, whereas the 2-ethyl-4-vinyl isomer **3a** crystallized as microscopic rectangular plates with micro-mp 224–226 °C.

Our results establish that the attachment of the porphyrin to the protein of *E. gracilis* cytochrome c_{558} is at the 4 position as in **16**. In addition, successful cleavage with sodium amalgam

16
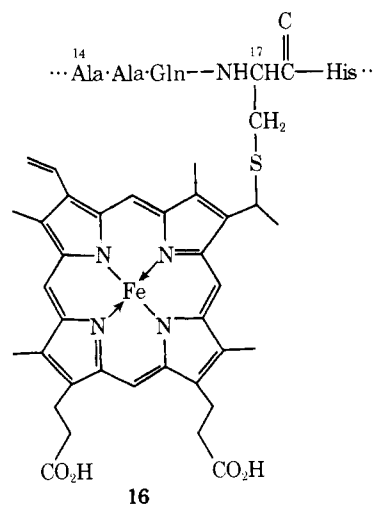
shows that the attachment is at the α carbon of the 4-ethyl group.[2] The presence of a free vinyl group has been confirmed and unambiguously assigned to the 2 position on the porphyrin. Finally, assuming the consistency of nature, these results indicate the sense of the porphyrin relative to the peptide in normal dithioether linked cytochromes *c* as having the 2 position of the porphyrin attached to the first (closest to the N

terminus) cysteine of the sequence ···Cys·X·Y·Cys·His··· and the 4 position attached to the later cysteine.

Experimental Section

Melting points are corrected and were taken in open capillary tubes on a Büchi melting point apparatus unless specified as micro-melting point in which case they were taken on a Kofler micro-hot-stage apparatus. Ultraviolet and visible spectra were taken on a Cary Model 14 spectrometer. NMR spectra were taken in $CDCl_3$ on Varian Model T-60 and HR-220 spectrometers using internal tetramethylsilane. Fourier-transform NMR were taken on a Bruker HXS-360 instrument at the Stanford Magnetic Research Laboratory. Mass spectra were obtained on a MS12 spectrometer. TLC analyses were performed on glass plates coated with Camag TLC silica gel. High-pressure liquid chromatograms were obtained on a Spectra Physics/Chromatronix Model 3500 liquid chromatograph employing a Model 770 variable wavelength detector set at 400 nm. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley.

Cultivation of Euglena. An inoculation sample of *Euglena gracilis klebs* variety *bacillaris* was obtained from the American Type Culture Collection (No. 10616) and was maintained by periodic reinoculation on agar slants. The *Euglena* was grown at 25–28 °C in sterilized media containing 8.7 g of dehydrated Difco *Euglena* broth and 10 g of glucose per liter.[25] In order to prevent contamination by cytochrome c_{552} light was excluded by wrapping the culture flasks in aluminum foil and keeping the culture room dark except when occupied. Initial cultures were started by inoculating 5 mL of media from agar slants. Near the end of log phase growth (ca. 5 days) these cultures were used to inoculate 100 mL of media. After another 5 days the 100-mL cultures were used to inoculate 1-L volumes of media and, finally, these were used to inoculate 11-L fermentors. Rotary shaking was used for all cultures except in the fermentor which employed stirring (150 rpm) and aeration (1500 mL/min). Cells were harvested with a Sharples continuous flow centrifuge. The yield was about 30 g/L of a compact paste, and the cells were frozen until use.

Cytochrome c_{558} Isolation. Frozen cells were thawed and lysed with a Bronwell Biosonik sonicator in 250-g batches in 8 mM P_i pH 7.2 buffer containing 0.2 M NaCl plus 25 g of fine glass beads (total volume was 900 mL), following cell rupture microscopically. After sonication the suspension was centrifuged; the supernatant was decanted, adjusted to pH 7.2, diluted to 12 L, and stirred twice with 25–30 g of preequilibrated Bio Rex 70 cation-exchange resin, Na^+ form. After settling the solution was decanted and the resulting orange-pink resin was loaded into a column and eluted with 0.5 M NaCl in 8 mM pH 7.2 P_i to give an orange solution. In this manner a solution containing 15.9 μmol [determined by the absorbance at 558 nm (ε 26 300)][6,8] of crude cytochrome c_{558} was obtained from 6.2 kg of *Euglena* cells. The cytochrome solution was divided into two equal portions which were purified separately.

The cytochrome solution was diluted with 15 vol of water, clarified by filtering twice through Whatman No. 1 paper, and then passed through a 2 × 10 cm column of Bio Rex 70 resin equilibrated with pH 7.2, 8 mM P_i buffer. The cytochrome, collected as a narrow band at the top of the column, was washed with 250 mL each of pH 7.2, 8 mM P_i and pH 7.2, 0.05 M P_i and eluted with 0.5 M pH 7.2 P_i to give a dark orange-red solution which was diluted to 500 mL with distilled water and the cytochrome was adsorbed onto a 2.5 × 25 cm column of equilibrated Bio Rex 70 resin. Elution with 8 mM pH 7.2 P_i buffer using an ionic strength gradient from 0 to 0.5 M NaCl removed an orange band which was diluted, 2 mL of a 0.01 M solution of K_3FeCN_6 was added, and the solution was allowed to stand 1 h to effect oxidation of the cytochrome. The cytochrome was again adsorbed onto a 2.5 × 25 cm column of resin, eluted with an ionic strength gradient, rediluted, reduced with $Na_2S_2O_4$, adsorbed onto a resin, and finally eluted as a narrow band with 8 mM pH 7.2 P_i containing 0.5 M NaCl. The cytochrome was stored in solution at 0 °C until used. Table I gives both the reported properties of *Euglena* cytochrome c_{558}[6,8] and those observed after the above purification.

Deuteroporphyrin dimethyl ester (10) was obtained in 39% yield [mp 221–223 °C (lit.[13] mp 222 °C)] from the resorcinol melt of hemin chloride (Sigma) as described;[13] an alternative procedure[29] gave 28% yield in our hands.

2- and 4-Acetyldeuteroporphyrin Dimethyl Ester (7a and 7b).[13] The copper complex of deuteroporphyrin dimethyl ester 10 was prepared

Table I. Properties of *Euglena gracilis* Cytochrome c_{558}

	Reported[6,8]			Obsd	
	λ	ϵ	Rel ϵ	λ	Rel ϵ
Mol wt	11 254				
Isoelectric point	9.59				
Spectral characteristics					
Reduced	558 (α)	26 000	1	558	1
	526	15 500	0.59	526	0.67
	422	158 000	6	422	6.1
Oxidized	530	10 500	0.4	530	0.4
	412	101 000	3.85	412	3.8
	280	30 000	1.13	280	1.32
A_{558} (red.)/A_{280} (ox.)			0.88		0.76

in 99% yield [mp 229–231 °C (lit.[13] mp 236 °C)] by the reported method[13] and 0.5 g (0.835 mmol) was acetylated as described,[13] except that the reaction was allowed to proceed for 30 s before adding water.

Chromatography on a 2 × 47 cm column of alumina (Woelm, neutral, activity III) with methylene chloride gave three bands plus a residue at the origin. Recrystallization of each of the three fractions from chloroform–methanol (1:3) gave 101 mg (22.5%) of deuteroporphyrin dimethyl ester (mp 221–223 °C), 253 mg (53%) of a mixture of monoacetyl derivatives 7a and 7b (NMR acetylmethyl singlets of approximately equal intensity at δ 3.1 and 3.15), and 60 mg (12%) of diacetyldeuteroporphyrin dimethyl ester [mp 238–241 °C (lit.[13] mp 240 °C)].

Column chromatography of the mixture of 7a and 7b on silica gel with methylene chloride[13] or methylene chloride with 0.3–0.5% 2-propanol failed to completely separate the two isomers. Chromatography on Camag TLC grade silica gel with the same solvents and with the aid of nitrogen pressure (8 lb) gave significantly enriched fractions; however, while the 2-isomer 7a was nearly completely insoluble in acetone, the 4-isomer 7b was freely soluble. Thus 1.09 g of a mixture of 7a and 7b was suspended in boiling acetone, cooled to 0 °C, and filtered. The precipitate was collected and treated again with acetone then recrystallized from chloroform–methanol (2:3) and chromatographed on 80 g of Camag TLC grade silica gel with methylene chloride containing 0.5% 2-propanol under 8 lb of pressure. Recrystallization of the first large fraction gave 511 mg (94% of the expected 50%, or 47%) of the 2-isomer 7a with mp 238.5–240.5 °C (lit.[16] mp 240 °C). The combined filtrates were evaporated to dryness, chromatographed, and recrystallized in the same manner to give 324 mg (60% of the expected 50%, or 30%) of the 4-isomer 7b as tiny needles, mp 211–213 °C (lit.[16] mp 212 °C, incorrectly designated[13] as the 2-isomer).

2- and 4-α-Hydroxyethyldeuteroporphyrin Dimethyl Ester (12a and 12b). 2-Acetyldeuteroporphyrin dimethyl ester (7a, 80 mg, 38 μmol) was dissolved in 50 mL of chloroform and stirred vigorously at room temperature while 50 mL of methanol and 750 mg (19.7 mmol) of $NaBH_4$ were added. The solution was stirred for 20 min and then 0.2 N HCl (95 mL) was added to pH 7, the layers were separated, and the red chloroform layer was washed with water, dried over sodium sulfate, filtered, and evaporated. The residue was chromatographed on a 2 × 19 cm column of alumina (Woelm, activity IV) with chloroform, and the major band was crystallized from chloroform–methanol (1:3) to give 74 mg (93%) of 12a: mp 236.5–239 °C (lit.[13] mp 240–242 °C; incorrectly designated as the 4-isomer).

The 4-isomer 12b was prepared in 92% yield in the same manner: mp 217–221 °C (lit.[13] mp 233–234 °C; incorrectly designated as the 2-isomer).

2- and 4-Vinyldeuteroporphyrin Dimethyl Ester [8a (Isopemptoporphyrin Dimethyl Ester) and 8b (Pemptoporphyrin Dimethyl Ester)]. A solution of 152 mg (800 μmol) of *p*-toluenesulfonic acid monohydrate in 125 mL of benzene was heated at reflux for 1 h under a Dean-Stark trap to remove the water; then 68 mg (120 mol) of 12a was added and the purple-red solution was heated at reflux for another 6 h. The solution was evaporated to dryness; the residue was dissolved in 35 mL of chloroform, washed twice with saturated sodium bicarbonate and once with water, dried over sodium sulfate, filtered, and evaporated. Chromatography on a 2 × 20 cm column of silica gel gave a major band which after crystallization from chloroform–methanol
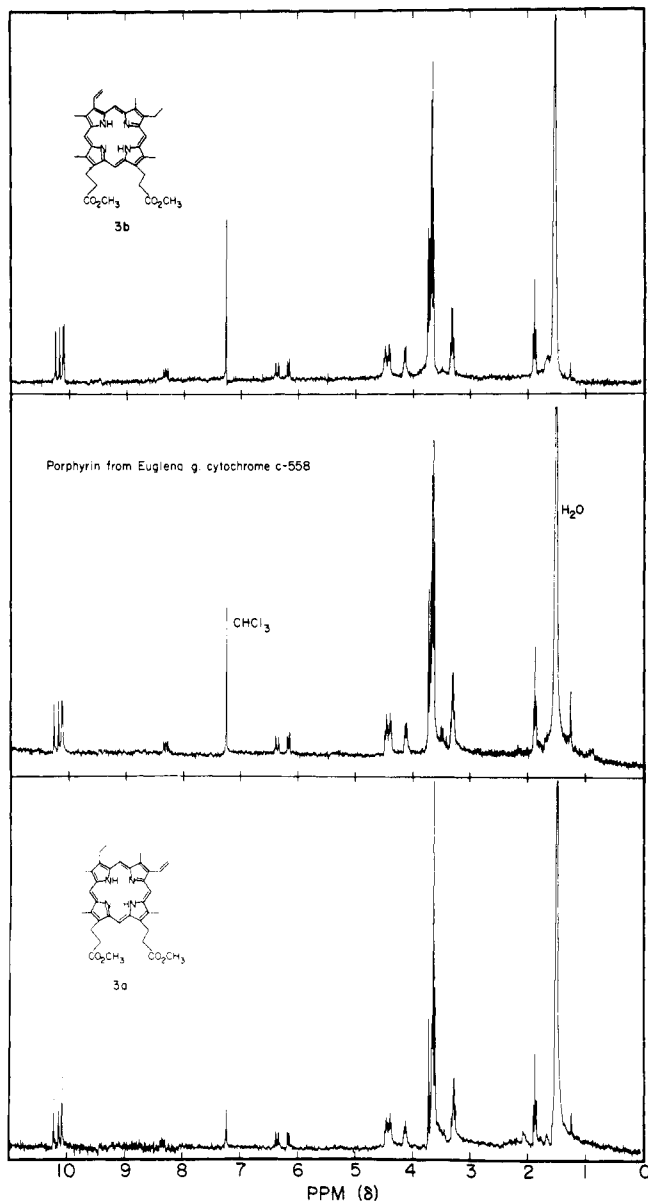
Figure 1. ^{1}H NMR spectra (360 MHz) of **3a**, of the porphyrin obtained from Na–Hg reductive cleavage of *E. gracilis* cytochrome c_{558} [*Euglena* (c_{558}) porphyrin], and of **3b**.

Figure 2. Expansion of the ^{1}H NMR spectra (360 MHz) in the OCH_3, ring CH_3 and CH_2, and meso-H regions.

(1:3) yielded 61 mg (80%) of isopemptoporphyrin methyl ester (**8a**): mp 218–221 °C (lit.[16] mp 219–221 °C); electronic and NMR spectra identical with those reported.[15,17] Anal. ($C_{34}H_{36}N_4O_4$) C, H, N.

For mass spectral analysis the copper complex was prepared by heating a solution of 5 mg (8.9 μmol) of **8a** and 5 mg of cupric acetate in 5 mL of $CHCl_3$ and 5 mL of CH_3OH at reflux for 1 h followed by cooling, filtration, and washing with methanol to give a bright red solid: MS m/e 627, 625 (M$^+$), 554, 552 (-$CH_2CO_2CH_3$), 481, 479 (-$2CH_2CO_2CH_3$).

The 4-vinyl isomer **8b** (pemptoporphyrin dimethyl ester) was prepared in 89% yield in the same manner: mp 208–210 °C (lit.[14,15] mp 209–210, 213–214 °C); electronic spectrum identical with that of its isomer **8a**; NMR spectrum extremely similar to that of **8a**; MS of copper complex identical with that of its isomer **8a**. Anal. ($C_{34}H_{36}N_4O_4$) C, H, N.

2- and 4-Ethyldeuteroporphyrin Dimethyl Ester (4a and 4b). Isopemptoporphyrin dimethyl ester (**8a**, 484 mg, 860 μmol) was hydrogenated at 30–35 °C for 45 min at atmospheric pressure in a solution containing 585 mg of poly(methyl methacrylate) (Aldrich, very high molecular weight), 113 mg of 30% Pd/C, and 90 mL of formic acid which had been freshly distilled from cupric sulfate.[26] The mixture was then poured into 450 mL of ether and the precipitated polymer and catalyst were removed, the insoluble material was washed with
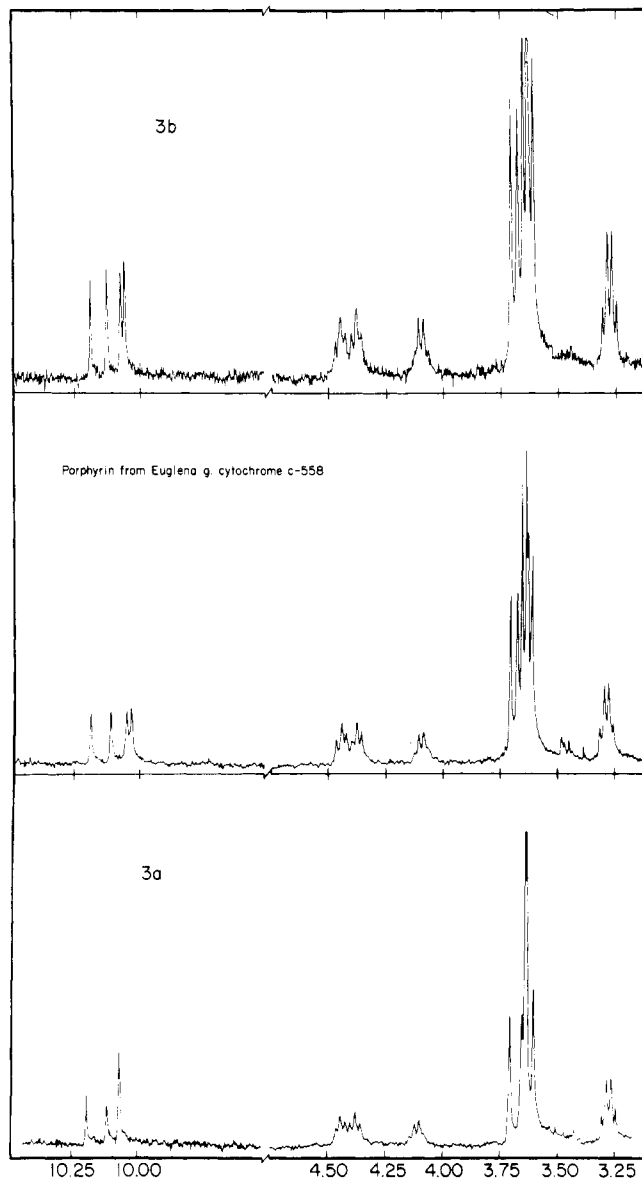
ether, and the porphyrin in the combined organic solvents was extracted with four 50-mL portions of 2.5% HCl. The acidic aqueous solution was then extracted with four 50-mL portions of chloroform, the combined red chloroform solution was washed with water, dried over sodium sulfate, filtered, and evaporated, and the residue was chromatographed on a 2 × 45 cm column of silica gel, eluting with chloroform. Evaporation of the major fraction gave 430 mg (89%) of **4a** which was crystallized from chloroform–methanol (1:3): mp 214–215.5 °C (lit.[27] mp 214 °C); copper complex, mp 229.5–231 °C (lit.[27] mp 230 °C); MS m/e 629, 627 (M$^+$).

The 4-isomer **4b** was prepared from **8b** in an identical manner in 85% yield: mp 213.5–215.5 °C (lit.[27,28] mp 213, 215–218 °C); mmp with **4a**, 178–195 °C; copper complex mp 228.5–230 °C (lit.[27] mp 220 °C); MS m/e 629, 627 (M$^+$).

2-Ethyl-4-acetyl- and 2-Acetyl-4-ethyldeuteroporphyrin Dimethyl Ester (13a and 13b). The copper complexes of **4a** and **4b** were acetylated by the same procedure used above for the monoacetylation of deuteroporphyrin dimethyl ester (**10**) except that the reaction was performed in methylene chloride for 5 min before quenching with water. The residue obtained was chromatographed on activity III alumina (Woelm) and the major band was crystallized. From the 2-ethyl isomer **4a** was obtained 78 mg (53%) of **13a**: mp 242.5–244 °C; NMR (CDCl$_3$, 0.05 M) δ 1.7 (t, 3 H), 3.1 (s, 3 H, -COCH$_3$), 3.4–3.7 (m, ring methyls, methyl esters, methylenes of propionic acid

side chains), 3.8–4.2 (m, methylenes α to ring of propionic acid side chains and $-CH_2CH_3$), 9.75 (m, 3 H), 10.7 (s, 1 H). Anal. ($C_{36}H_{40}H_4O_5$) C, H, N. Copper complex: mp 267–269 °C; MS m/e 671 (M^+).

The isomer **13b** was prepared in the same manner in 52% yield, mp 256–258 °C (lit.[27] mp 261 °C). Both isomers behaved the same on TLC: R_f 0.36, silica gel–methylene chloride with 3% ethyl acetate.

2-Ethyl-4-α-hydroxyethyl- and 2-α-hydroxyethyl-4-ethyldeuteroporphyrin dimethyl ester (14a and 14b) were prepared by a procedure identical with that used for the mono-α-hydroxyethyl isomers **12a** and **12b**. Compound **14a** was obtained from **13a** in 90% yield after recrystallization: mp 208–210 °C; electronic spectrum (CHCl₃) λ (rel absorbance), 402 (13.3), 499 (1), 535 (0.72), 568 (0.49), 595 (0.095), 622 (0.305); TLC (silica gel, CHCl₃ with 7% CH₃OH) R_f 0.55; TLC (CHCl₃ with 20% ethyl acetate) R_f 0.44. Anal. ($C_{36}H_{42}N_4O_5$) C, H, N. Copper complex: mp 196–198.5 °C; MS m/e 655, 653 ($M^+ - H_2O$).

The 2-α-hydroxyethyl-4-ethyl isomer **14b** was obtained in 94% yield: mp 201–203 °C; electronic spectrum (CHCl₃) λ (rel absorbance) 402 (13.1), 500 (1), 537 (0.69), 568 (0.49), 596 (0.092), 623 (0.30); TLC identical with that of **14a**; MS m/e 592 ($M^+ - H_2O$). Anal. ($C_{36}H_{42}N_4O_5$) C, H, N. Copper complex: mp 206–208 °C; MS m/e 655, 653 ($M^+ - H_2O$).

2-Ethyl-4-vinyl- and 2-vinyl-4-ethyldeuteroporphyrin dimethyl ester (3a and 3b) were prepared by the dehydration of **14a** and **14b** with p-toluenesulfonic acid as described for the monovinyl isomers **8a** and **8b**. Compound **3a** was obtained in 82% yield as microscopic rectangular plates: micro-mp 224–226 °C (sintered at 219 °C); electronic spectrum (CH₂Cl₂) λ (rel absorbance) 403 (13.6), 503 (1), 539 (0.93), 571 (0.54), 625 (0.29); TLC (silica gel, CH₂Cl₂ with 1% CH₃OH) R_f 0.38. Anal. ($C_{36}H_{40}N_4O_4$) C, H, N. Copper complex: MS m/e 655, 653 (M^+).

The 2-vinyl-4-ethyl isomer **3b** was obtained in 79% yield as microscopic needles: micro-mp 232–234 °C; electronic spectrum (CH₂Cl₂) λ (rel absorbance) 402.5 (13.2), 503 (1), 539 (0.92), 571 (0.52), 625 (0.28); TLC identical with that of **3a**. Anal. ($C_{36}H_{40}N_4O_4$) C, H, N. Copper complex: mp 232–234 °C; MS m/e 655, 653 (M^+).

Cleavage of Porphyrin from *Euglena* Cytochrome c₅₅₈. *Euglena gracilis* cytochrome c_{558} (3.3 μmol) in a solution of 8 mM pH 7.2 P_i containing 0.5 M NaCl (13 mL total) was purged with nitrogen and heated to 80 °C (bath). With vigorous stirring 12 g of powdered 3% sodium amalgam was added all at once. After stirring in the dark for 2 min the colorless solution was decanted from mercury and excess amalgam into a separatory funnel containing 25 mL of ether with 20% acetic acid which had been purged with nitrogen, and the reaction vessel was rinsed twice with water which was also added to the separatory funnel. Freshly recrystallized DDQ (from methylene chloride, 3.0 mg, 13.2 μmol) was added and the mixture was shaken vigorously to give a deep red organic layer. After 30 s, 200 mg of $Na_2S_2O_4 \cdot 2H_2O$ was added and after vigorous shaking, the layers were separated, the aqueous was extracted with 25 mL of ether, and the combined ethereal extracts were evaporated. The residue was esterified at 0 °C for 18 h in 25 mL of methanol containing 1 mL of $HC(OCH_3)_3$ and 5% H_2SO_4 (by weight), and the mixture was poured into 20 mL of chloroform and washed with 2 vol of water and 2 vol of 5% sodium bicarbonate. Evaporation followed by chromatography on alumina (Woelm, activity III, with methylene chloride–benzene, 1:1) gave a red fraction (22% yield), and a second porphyrinic fraction was obtained by elution with 2% CH₃OH in CH₂Cl₂.

The first fraction was combined with similar fractions obtained from previous reactions (10.5% yield) in which air oxidation (4–8 h) had been used after the sodium amalgam reduction. The combined material (1.1–1.2 μmol) was chromatographed on a 1.5 × 12 cm column of silica gel with CH₂Cl₂ which removed a trace of material with an absorbance at 662 nm. High-pressure liquid chromatography (Lichrosorb, 250 mm × ⅛ in. column, CH₂Cl₂ containing 1.5–2% 2-propanol) revealed this porphyrin as a mixture of monovinylmonoethyldeuteroporphyrin dimethyl ester **3a** or **3b** (70%; retention time of 4 min with a flow rate of 0.8 mL/min) and mesoporphyrin dimethyl

ester (**2**, 30%; retention time of 6.5 min) plus a small amount of unidentified material. A control mixture of synthetic **3a** (70%) and the dimethyl ester of **2** (30%) had an identical electronic spectrum and LC chromatogram. Column chromatography of the *Euglena* (c_{558}) porphyrin mixture on Camag TLC grade silica gel with methylene chloride containing 0.1–0.3% 2-propanol under 8 lb of nitrogen pressure gave a fraction which by LC was shown to be monovinyl-monoethyldeuteroporphyrin dimethyl ester **3a** or **3b** with only a trace (<2%) of mesoporphyrin dimethyl ester (**2**).

The purified fraction (0.41 μmol, 240 μg, assuming $\epsilon = 14\ 000$ at 503 nm) had an electronic spectrum essentially identical with the synthetic isomers **3a** and **3b**: λ (rel absorbance in CH₂Cl₂) 503 (1), 539–540 (0.9), 571 (0.53), 625–626 (0.28). A 360-MHz NMR spectrum of the residue was taken at 1 mM in CDCl₃ (100 atom % D, Bio-Rad) which was first washed with D₂O (99.8 atom % D) and distilled under N₂. Comparison with the spectra taken of **3a** and **3b** under identical conditions revealed the identity of the *Euglena* (c_{558}) porphyrin with 2-vinyl-4-ethyldeuteroporphyrin dimethyl ester **3b** (Figures 1 and 2).

After recording the NMR spectra, the *Euglena* (c_{558}) porphyrin in CDCl₃ was added to 3 mL of boiling methanol and cooling at −25 °C gave microscopic needles, identical with those of **3b**: micro-mp 231–233 °C.

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