Bile Pigment–Protein Interactions. Coupled Oxidation of Cytochrome *c*†

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ABSTRACT: A new methodology is described for the chemical modification of the heme prosthetic group of horse heart cytochrome *c*. The selective modification of the heme moiety of cytochrome *c* is facilitated by utilizing coupling oxidation conditions. Comparison of the absorption spectra of this chemically modified cytochrome *c* species in two different solvents (aqueous pyridine and carbon monoxide saturated 6 M guanidinium chloride) with those of two model compounds [bis(pyridine)(2,3,7,8,12,13,17,18-octaethyl-5-oxaporphyrinato)iron(II) tetrafluoroborate salt and (pyridine)carbonyl(2,3,7,8,12,13,17,18-octaethyl-5-oxaporphyrinato)iron(II) tetrafluoroborate salt] shows that coupled oxidation of cytochrome *c* affords a new protein with a covalently bound iron(II) oxaporphyrin prosthetic group. Amino acid analysis of this protein-bound iron(II) oxaporphyrin species reveals that only limited modification of the primary structure of the apoprotein occurs during coupled oxidation of cytochrome *c*. This protein-bound iron(II) oxaporphyrin species is also interconvertible to a protein-bound bilatriene species under hydrolytic conditions. The synthetic utility of the coupled oxidation of cytochrome *c* for the preparation of chromoproteins which possess covalently bound iron(II) oxaporphyrin and bilatriene prosthetic groups is considered.

The photochemical properties of linear tetrapyrrolic pigments are significantly influenced by their association with proteins. In solution, linear tetrapyrroles are relatively inert photochemically, typically exhibiting low fluorescence quantum yields ($\phi_{Fl} < 10^{-3}$), due to efficient radiationless de-excitation pathways (Scheer, 1981). However, when bound to native biliproteins, as in the photosynthetic light harvesting phycobiliproteins and in phytochrome, the photoreceptor which mediates photomorphogenesis in plants, the de-excitation pathways of these pigments are significantly altered. Isolated phycobiliproteins exhibit fluorescence with quantum yields approaching unity, whereas the photointerconversion of the two forms of phytochrome ($P_R \rightleftharpoons P_{FR}$) displays quantum yields between 0.13 and 0.17 at room temperature (Scheer, 1981). The differences between the photochemical properties of the phycobiliproteins and phytochrome must be primarily due to the noncovalent chromophore–protein interactions, since the prosthetic group of P_R phytochrome (**1**) and at least one of the three bilin moieties of C-phycocyanin (**2**) (both in denatured state) have very similar dihydrobilatriene prosthetic groups and identical covalent linkages between bilin and apoprotein (Lagarias & Rapoport, 1980; Lagarias et al., 1979). Our understanding of the noncovalent chromophore–protein interactions in native biliproteins is minimal since their X-ray crystal structures have not yet been determined.

An alternate approach to elucidate the relationship between protein–bilatriene interactions and the photochemical properties of the bilin prosthetic groups would be the synthesis of small biliproteins whose primary through quaternary structure could be carefully controlled. Few proteins have been as extensively characterized as have the hemoproteins. For this reason, the suitability of heme protein precursors for the synthesis of artificial biliproteins was first considered. It is well established that heme compounds are susceptible to oxidative attack at the meso positions in the presence of reducing agents such as ascorbate [see reviews by Lemberg (1956) and O'Carra (1975)]. Since such "coupled oxidation" proceeds with the cleavage of the porphyrin ring and formation of open-chain tetrapyrroles, its utility as a chemical model for the biogenesis of naturally occurring, linear tetrapyrroles has gained widespread acceptance (Lemberg, 1956; O'Carra, 1975; Schmid & McDonagh, 1975). Although many coupled oxidation studies of heme proteins have been reported, the synthesis of biliprotein complexes of sufficient stability and quantity to facilitate biophysical and biochemical analysis has not heretofore been realized with this method. The utility of the coupled oxidation method for the preparation of new biliproteins, therefore, was determined in the present study. Cytochrome *c* was chosen for the first studies for several reasons: (1) the primary sequences (Borden & Margoliash, 1976; Dickerson & Timkovich, 1976; Ambler, 1976) and X-ray structures (Timkovich, 1979) for numerous *c*-type cytochromes are known; (2) cytochrome *c* is available in quantity from many different organisms; (3) the heme moieties of *c*-type cytochromes are covalently attached to the apoproteins by one or two α-thioether linkages (as illustrated for the heme undecapeptide **3** from horse heart cytochrome *c*) in a manner analogous with the bilatriene–apoprotein linkages found in the biliproteins phytochrome (**1**) and C-phycocyanin (**2**) (Scheer, 1981; Lagarias & Rapoport, 1980; Lagarias et al., 1979); and (4) coupled oxidation of cytochrome *c* provides a potential method to enable the comparative investigation of different bilatriene–protein interactions which arise from cleavage at each of the four methine bridges of the heme prosthetic group (O'Carra, 1975).

Previous studies have indicated that the heme prosthetic group of cytochrome *c* is stable to treatment with ascorbic acid and molecular oxygen (Kench, 1954; Westman, 1975). In view of our present understanding of the mechanism of the coupled oxidation, this unreactivity of the heme prosthetic group of cytochrome *c* can be rationalized in terms of the nonavailability of a free coordination site at the heme iron for O_2 binding (Lemberg, 1956; O'Carra, 1975; Brown, 1976). Because of the presence of a hexacoordinate iron ligand sphere, with side chains of methionine and histidine providing the two axial ligands, native cytochrome *c* exhibits no O_2 binding. Oxygen has been shown to directly interact with the heme iron of cytochrome *c*, however, when one (or both) of the axial ligands is removed or altered by chemical modification (Schejter & Aviram, 1970). It is then conceivable that if conditions could be found where the heme iron were made accessible to mo-

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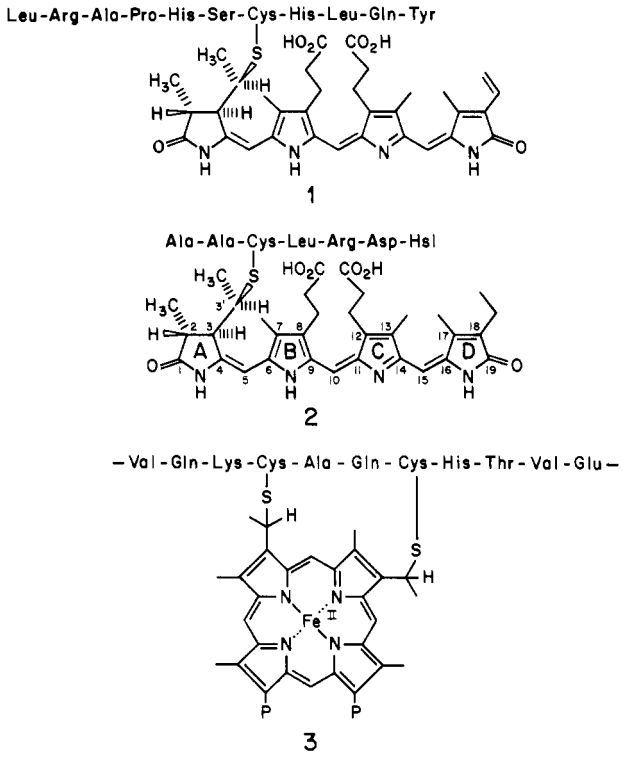


lecular oxygen binding, coupled oxidation of cytochrome *c* would necessarily follow. The present work was therefore undertaken to establish conditions which facilitate the coupled oxidation of the heme prosthetic group of horse heart cytochrome *c* and to provide the preliminary chemical and spectroscopic characterization of the new protein products.

Materials and Methods

Reagents. Horse heart cytochrome *c* (Sigma, type VI) was used for the coupled oxidation studies. L(+)-Ascorbic acid and guanidine hydrochloride (grade I) were obtained from Sigma. Pyridine was distilled from barium oxide (BaO) and ninhydrin. Deionized water, purified with a Milli Q purification system (Millipore Corp.), was used for buffers. Bis(pyridine)(2,3,7,8,12,13,17,18-octaethyl-5-oxaporphyrinato)iron(II) tetrafluoroborate salt (**6**) ($R_1 = R_2 = R_3 = Et$)[1] and (pyridine)carbonyl(2,3,7,8,12,13,17,18-octaethyl-5-oxaporphyrinato)iron(II) tetrafluoroborate salt (**6**) ($R_1 = R_2 = R_3 = Et$) were synthesized as previously described (Lagarias, 1982). Octaethylbiliverdin (**8**) ($R_1 = R_3 = Et$) was prepared according to a modification of the literature method (McDonagh, 1979; Lagarias, 1982). Sephadex G 10-120 (40–120 mesh, Pharmacia) was used for gel filtration. All other reagents, chemicals, and gases were reagent grade.

Spectroscopic Measurements. UV–visible absorption spectra were recorded with a Hewlett-Packard 8450A spectrophotometer. For kinetic absorbance measurements, a continuous flow apparatus for spectrophotometric monitoring of the reaction mixture with the HP 8450A instrument was designed. With this modification, the reaction mixture was continuously monitored by its recirculation through a flow-through cuvette (0.1-cm path length) by using a peristaltic pump. A 2-s sampling time at 10-s intervals was used for these measurements.

General Procedures. All coupled oxidations, chromatographic separations, and subsequent hydrolyses were performed under reduced light. Amino acid analyses were performed on a Durrum D500 amino acid analyzer at the Protein Structure Laboratory, University of California, Davis, CA.

[1] Abbreviations: Et, ethyl; CO, carbon monoxide; N_2, nitrogen.

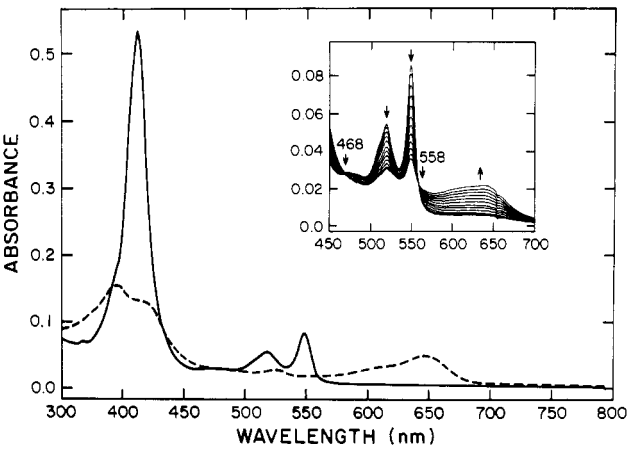


FIGURE 1: Coupled oxidation of horse heart cytochrome *c*. (—) Horse heart cytochrome *c* [$c = 3.93 \times 10^{-5}$ M in 8.37×10^{-3} M L(+)-ascorbic acid in 50% pyridine/water under an N_2 atmosphere]; (---) same solution after oxygenation for 12 min (O_2 flow rate 15 mL/min). Inset: Spectral measurements in the region 450–700 nm taken in 10-s intervals from $t = 0$ to $t = 120$ s where $t = 0$ represents the introduction of O_2 into the cytochrome *c* solution.

Coupled Oxidation of Cytochrome c. Preparation of the (Oxaporphyrin)iron(II)–Cytochrome c Apoprotein Complex. A solution of horse heart cytochrome *c* (20 mg) in 20 mL of deionized water was degassed with N_2 for 20 min whereupon 20 mL of pyridine was added with stirring under N_2. After an additional 10-min N_2 degassing, a freshly prepared aqueous solution of ascorbic acid (1.0 mL, $c = 0.34$ M) was introduced to the reddish brown solution with stirring. After 15 min of equilibration with stirring under N_2, when the absorbance at 412, 518, and 548 nm remained constant, the nitrogen flow was replaced with O_2. During subsequent oxygenation (flow rate of 15 mL/min), the reaction mixture was monitored spectrophotometrically at 10-s intervals. When the increase of the absorbance at 650 nm was no longer observed, the O_2 stream was replaced with N_2, the reaction mixture was sparged for an additional 10 min, and 160 mL of acetone was added with stirring. After 20 min, the green precipitated protein was collected by centrifugation (10 min, 7000*g*), washed twice with acetone, and dried in vacuo to give a green powder weighing 17.8 mg (89%).

Hydrolysis of the (Oxaporphyrin)iron(II)–Cytochrome c Apoprotein Complex. To a solution of the coupled oxidized cytochrome *c* product (5.8 mg) in 10 mL of deionized water was added 4 mL of 20% (v/v) formic acid/water. The mixture was heated at 50 °C under N_2. After 5 h, when the decrease in absorbance at 395 nm was no longer observed, the blue reaction mixture was concentrated to 2 mL in vacuo and diluted to 5 mL with deionized water, and the protein was precipitated with acetone (80 mL). The blue pellet was collected by centrifugation (10 min, 7000*g*), washed twice with acetone, and dried under nitrogen to give a blue solid weighing 3.8 mg (66%).

Results

As shown in Figure 1 (solid line), cytochrome *c* was initially prereduced to the hemochrome with a 300-fold excess of ascorbic acid in a 50% (v/v) pyridine/water mixture under N_2. During subsequent oxygenation, the reaction was accompanied by a marked reduction of the hemochrome absorption maxima at 418, 520, and 548 nm and an increase in absorbance be-

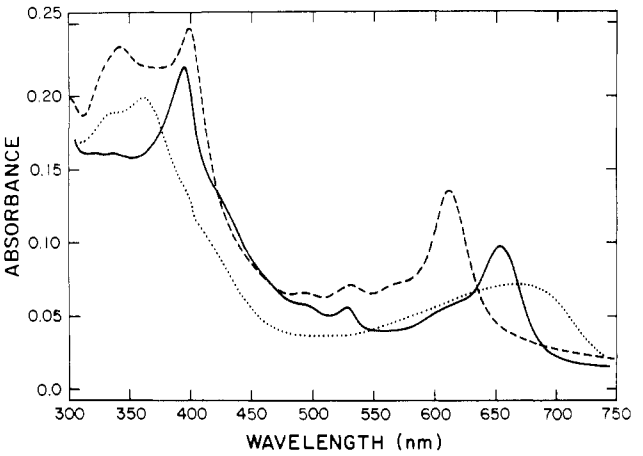


FIGURE 2: Absorption spectra of the coupled oxidation product of cytochrome *c* (l = 1.0 cm). (—) c = 1.00 mg in 10 mL of water; (---) c = 1.23 mg in 10 mL of CO-saturated 6.0 M guanidinium chloride; (···) c = 1.00 mg in 10 mL of 5% HCOOH after hydrolysis for 5 h at 50 °C.

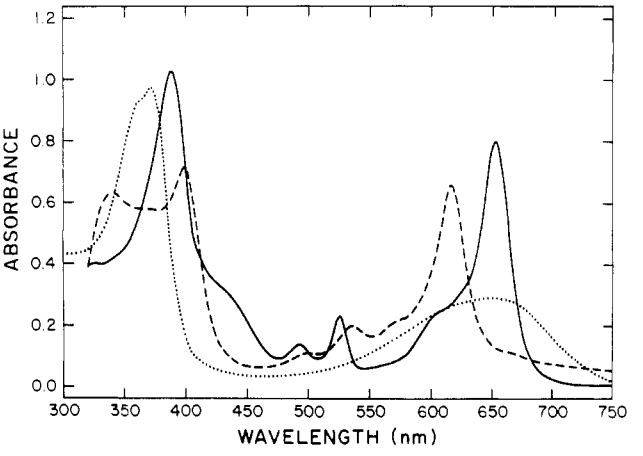


FIGURE 3: Absorption spectra of the coupled oxidation products of octaethylheme **4** ($R_1 = R_2 = R_3$ = Et) (l = 0.3 cm). (—) **6** ($R_1 = R_2 = R_3$ = Et; axial ligands, both pyridines; $c = 6.5 \times 10^{-5}$ M in pyridine); (---) **6** ($R_1 = R_2 = R_3$ = Et; axial ligands, CO and pyridine; $c = 6.5 \times 10^{-5}$ M in CO-saturated $CHCl_3$); (···) **8** ($R_1 = R_2 = R_3$ = Et; $c = 6.5 \times 10^{-5}$ M in $CHCl_3$).

tween 630 and 640 nm. As shown in the insert to Figure 1, this initial process exhibited well-defined isosbestic points at 468 and 558 nm. Furthermore, difference measurements showed that an intermediate with absorption maxima at 424 and 636 nm was produced during this initial process (results not shown).

After these initial spectral changes which occurred within the first 3 min upon oxygenation, a secondary reaction commenced. This reaction was accompanied by a further increase in absorbance above 640 nm and the loss of the isosbestic points. Concomitant with this increase in absorbance at 642 nm was the appearance of a new peak at 395 nm. The absorbance at 395 nm continued to increase for an additional 10 min and then reached a constant value. Addition of more ascorbic acid at this point had no further effect on the absorbance of the reaction mixture. The final absorption spectrum of the reaction mixture is shown in Figure 1 (dashed line). The protein product was then isolated by precipitation with 80% (v/v) acetone and collected by centrifugation. Subsequent spectrophotometric examination of the supernatant showed that >95% of the green pigment remained associated with the protein precipitate. Gel filtration of the green protein residue (Sephadex G10, eluant 50% (v/v) pyridine/water) also confirmed the presence of a covalent linkage between pigment and protein.

The green protein product was first characterized by comparative absorption spectroscopy with the previously characterized (oxaporphyrin)iron(II) model compound **6** ($R_1 = R_2 = R_3$ = Et), prepared by coupled oxidation of octaethylheme **4** ($R_1 = R_2 = R_3$ = Et) (Lagarias, 1982). In Figures 2 and 3 (solid lines), an aqueous solution of the green coupled oxidation product of cytochrome *c* and a pyridine solution of the iron(II) oxaporphyrin model compound **6** ($R_1 = R_2 = R_3$ = Et) are compared. Both compounds exhibit intense absorption maxima at 395 and 654 nm with minor absorption maxima at 490 and 528 nm, which are characteristic of a species known as a verdohemochrome (Levin, 1966; Jackson et al., 1968; Clezy & Liepa, 1970; Bonnett & Dimsdale, 1972; Lagarias, 1982). Additionally, upon CO treatment, both verdohemochrome species displayed nearly identical spectral changes (Figures 2 and 3, dashed lines). These changes included the appearance of a new absorption maximum at 610–615 nm, the loss of the absorption maximum at 652 nm, and the "apparent" splitting of the absorption maximum at 395 nm into two new maxima at 342 and 399 nm. For the protein-bound verdohemochrome species, these CO-induced spectral changes required prior unfolding of the protein with 6 M guanidinium chloride. In the absence of a protein denaturant, these spectrophotometric changes were not effected by CO treatment.

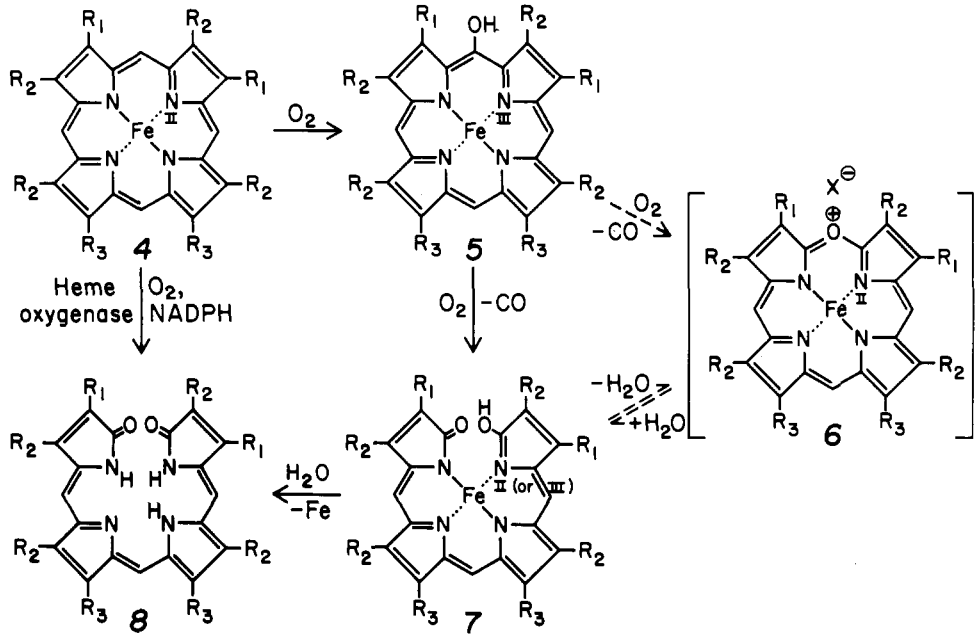
Table I: Amino Acid Analysis of Horse Heart Cytochrome *c* before and after Coupled Oxidation

	cytochrome *c* [a]		coupled oxidation product,[b]
amino acid	calcd no. of residues	found no. of residues[c]	found no. of residues[d]
Asp	8	8.0	7.9
Thr	10	9.1	8.9
Glu	12	12.0	12.0
Pro	4	5.4	4.0
Gly	12	11.8	11.6
Ala	6	6.0	6.9
Cys	2	0.4	1.2
Val	3	3.1	3.4
Met	2	1.9	1.5
Ile	6	5.6	5.4
Leu	6	6.0	5.7
Tyr	4	3.8	3.8
Phe	4	3.9	3.7
His	3	2.9	2.8
Lys	19	18.5	17.2
Arg	2	1.5	2.0

[a] Hydrolyzed 267 μg of horse heart cytochrome *c* (Sigma type VI) in 1.0 mL of 6 N HCl (pierce) + 50 μL of 5% phenol, twice freeze–thaw degassed in vacuo and sealed under vacuum, for 21 h at 110–115 °C. After being dried, the hydrolysate was dissolved in 580 μg of citrate buffer, pH 4.0, and 20 μL was applied to the amino acid analyzer. [b] Hydrolyzed 290 μg of coupled oxidation product of horse heart cytochrome *c* as above. After being dried, the hydrolysate was dissolved in 600 μL of buffer, and 20 μL was applied to the amino acid analyzer. [c] Normalized to 12.0 glutamic acid residues (found, 0.70 nmol/residue). [d] Normalized to 12.0 glutamic acid residues (found, 0.74 nmol/residue).

As a measure of nonspecific chemical modification of the amino acid side chains of cytochrome *c*, the amino acid composition of the protein was determined before and after coupled oxidation (Table I). Of the amino acids which are particularly sensitive toward oxidation, the side chain of methionine appeared to be the only residue significantly modified. A 25% reduction of methionine was observed as a result of coupled oxidation of cytochrome *c*. In addition to methionine, lower recoveries of proline and lysine, 75% and 93%, respectively,

Scheme I: Proposed Intermediates in the in Vivo Catabolism of Heme[a]



[a] The oxaporphyrin-iron complex (8) is the major product of in vitro coupled oxidation of heme compounds.

were also observed after coupled oxidation. In repetitive experiments, the loss of lysine was consistently observed. Quantitation of proline was made considerably more difficult due to chromophore breakdown products (unpublished results).

In addition to the similar spectral and ligand binding properties of the iron(II) oxaporphyrin model compound **6** ($R_1 = R_2 = R_3 = Et$) and the coupled oxidation product of cytochrome *c*, both compounds were converted to similar species under "hydrolytic" conditions. "Hydrolysis" of the model compound (**6**) ($R_1 = R_2 = R_3 = Et$) required the addition of an oxidizing agent to afford octaethylbiliverdin (**8**) ($R_1 = R_2 = R_3 = Et$) as the major product (Lagarias, 1982; Figure 3, dotted line). Conversion of the coupled–oxidized cytochrome *c* product to a species with an absorption spectrum similar to that of octaethylbiliverdin (**8**) ($R_1 = R_2 = R_3 = Et$) was accomplished with aqueous formic acid alone (Figure 2, dotted line). Evidence that the pigment prosthetic group remained covalently linked with the cytochrome *c* apoprotein was confirmed by coprecipitation and gel filtration experiments.

Discussion

In contrast to the previous studies (Kench, 1954; Westman, 1975), the present results show that coupled oxidation of the heme prosthetic group of cytochrome *c* is accomplished with ascorbic acid and molecular oxygen. This reaction requires large amounts of pyridine (i.e., 50% by volume) to proceed effectively. In the absence of pyridine or in the presence of small amounts of pyridine (<1% by volume), coupled oxidation of cytochrome *c* was not observed. Only oxidation–reduction of the heme iron of cytochrome *c* by ascorbic acid and O_2 was observed by utilizing these low pyridine concentrations. We believe, therefore, that the major role of pyridine is to deform the tertiary structure of cytochrome *c*. As a consequence of this tertiary structural change, the heme iron is made more accessible to axial coordination of molecular oxygen—a prerequisite for coupled oxidation. Alkylation of methionine-80 of cytochrome *c* (Shejter & Aviram, 1970) also renders the heme moiety susceptibile to coupled oxidation at significantly lower pyridine concentrations (L. Payne and J. C. Lagarias, unpublished experiments).

The present spectrophotometric analysis of the coupled oxidation of cytochrome *c* concurs with the generally accepted pathway of in vivo and in vitro heme oxidation shown in Scheme I (Schmid & McDonagh, 1975; Kikuchi & Yoshida, 1980; Lagarias, 1982). The initial spectrophotometric changes are consistent with the initial meso hydroxylation of the heme prosthetic group of cytochrome *c* (insert to Figure 1). Spectrally similar *meso*-hydroxyheme (or iron oxophlorin) species of general structure **5** have been obtained from treatment of numerous hemochromes with hydrogen peroxide (Libowitzsky, 1940; Stier, 1942; Jackson et al., 1968; Clezy & Liepa, 1970; Bonnett & Dimsdale, 1972). Consonant with these literature observations, the nascent protein-bound iron oxophlorin intermediate, shown in the present study to display absorption maxima at 424 and 636 nm, gives rise to a new species known as a verdohemochrome upon further oxygenation. The elucidation of the iron(II) oxaporphyrin structure **6** for the verdohemochrome derived from coupled oxidation of octaethylheme **4** ($R_1 = R_2 = R_3 = Et$) was recently established (Lagarias, 1982). As shown in Figure 3 (solid and dashed lines), the absorption spectral characteristics of this iron(II) oxaporphyrin model compound (**6**) ($R_1 = R_2 = R_3 = Et$) are significantly influenced by the axial ligands. Carbon monoxide replacement of one of the two pyridine axial ligands of the model verdohemochrome species **6** ($R_1 = R_2 = R_3 = Et$) results in a large change in its electronic spectrum—characteristic of an iron(II) oxaporphyrin system (Lagarias, 1982). Because of the analogous spectral changes which occur when the coupled oxidation product of cytochrome *c* is treated with carbon monoxide, the assignment of the iron(II) oxaporphyrin structure (**6**) (R_1 = cytochrome *c* apoprotein via two thioether linkages, $R_2 = CH_3$, R_3 = propionic acid; only one oxaporphyrin isomer is shown) to its prosthetic group is made.

Although the similarities between the absorption spectra of this protein-bound iron(II) oxaporphyrin species and the model iron(II) oxaporphyrin species (**6**) ($R_1 = R_2 = R_3 = Et$) have been emphasized, notable differences are evident. These differences pertain to the relative extinction coefficients of the blue and red absorption maxima. Differences in axial ligands

could provide one possible explanation for these spectral differences between these two iron oxaporphyrin species. The exact nature of the axial ligand(s) for the protein-bound iron(II) oxaporphyrin species is unknown at present. Since the formation of the blue monocarbonyl species requires prior unfolding of the protein with guanidinium chloride, we believe either that the apoprotein must provide two ligands to complete the hexacoordinate ligand sphere of the iron atom or that it prevents access of CO to the iron oxaprophyrin moiety. Besides differences in axial ligands, other factors may also contribute to the observed spectral differences between the model and protein-bound iron(II) oxaporphyrin species. These include differences in the oxaporphyrin counterion X^-, differences in solvation of the oxaporphyrin prosthetic group, and/or differences due to the presence of a mixture of oxaporphyrin isomers resulting from cleavage at the α-, β-, γ-, or δ-meso positions of the asymmetric heme moiety of cytochrome *c* (O'Carra, 1975).

Previous studies have shown that bis(pyridine)iron(II) oxaporphyrins (verdohemochromes) undergo facile hydrolytic interconversions to bilatriene species of general structure **8** (Lemberg, 1956; Levin, 1966; Bonnett & Dimsdale, 1972; McDonagh, 1979). As shown in Figure 2, mild acid hydrolysis of the protein-bound, iron oxaporphyrin species affords a new species which displays two broad absorption maxima at 350–370 and 650–680 nm. These spectrophotometric properties are characteristic of a fully conjugated linear tetrapyrrole such as octaethylbiliverdin (**8**) ($R_1 = R_2 = R_3 =$ Et). This comparative spectrophotometric analysis (Figures 2 and 3, dotted lines) therefore reveals that acid treatment of the protein-bound iron(II) oxaporphyrin predominantly yields a bilatriene chromophoric species. In contrast, the interconversion of the iron(II) oxaporphyrin model compound (**6**) ($R_1 = R_2 = R_3 =$ Et) to octaethylbiliverdin (**8**) ($R_1 = R_2 = R_3 =$ Et) requires an oxidizing agent (Lagarias, 1982). Whether molecular oxygen or various oxidized species of ascorbic acid participate in this hydrolysis is presently under investigation.

Reduced molecular oxygen species such as superoxide, hydroxyl radicals, and peroxide are known to be formed in solutions of ascorbic acid and molecular oxygen (Sawyer et al., 1980). These species are therefore likely to be present under the coupled oxidation conditions employed in this study. In addition, the formation of these activated oxygen species via heme iron facilitated electron transfer pathways to molecular oxygen is also probable (Bonnett, 1981). Despite the presence of these oxidizing species during coupled oxidation, nonspecific chemical modification of the cytochrome *c* apoprotein is minimal and restricted to a few amino acid residues—methionine, lysine, and proline. Under the reaction conditions used in this study, conversion of the methionine sulfur to a sulfoxide or sulfone is a reasonable explanation for the lower recoveries of this amino acid. Two other thioethers—those involved in the linkages between the apoprotein and prosthetic group—may be similarly modified during coupled oxidation. The extent of this modification was not determined in the present study. The degree of sulfur atom oxidation as well as the possibility of reversing such modification by reductive means is presently under unvestigation. In addition to sulfur atom modifications of cytochrome *c*, low recoveries of both lysine and proline were observed after coupled oxidation of cytochrome *c*. With regard to this observation, preliminary studies on the heme undecapeptide **3** have shown that the side chain of the lysine-13 at the heme binding site is significantly modified during coupled oxidation (unpublished results). The possibility of the participation of an "activated" molecular oxygen–heme species in this unusual side reaction is implicated by these results.

Despite these nonspecific chemical modifications, no major modification of the primary structure of cytochrome *c* was observed during its coupled oxidation. These results suggest that the coupled oxidation holds great promise for the semisynthesis of two new classes of proteins with covalent bound iron(II) oxaporphyrin and biliverdin prosthetic groups. Because of the presence of a new positive charge in the protein-bound iron(II) oxaporphyrin species and the removal of the iron moiety (and its associated axial ligands with the apoprotein) in the biliverdin-bound species, the chemical modification of the heme prosthetic group of cytochrome *c* described in this study undoubtedly has a significant influence on the tertiary structure of this chromoprotein. The nature of the modification in the tertiary structures of these chemically modified species will be addressed in a future study.

In conclusion, the present study shows that the *c*-type heme prosthetic group undergoes facile cleavage to protein-bound iron(II) oxaporphyrin and bilatriene species by means of the coupled oxidation reaction. In view of these results, it is intriguing to consider whether an oxidative cleavage of the heme prosthetic group of cytochrome *c* (or an analogous heme protein) would be a mechanism which operates in vivo. The metabolic fate of the heme moiety of cytochrome *c* during its degradation in vivo is unknown (Maines, 1977). In addition, the mechanisms of posttranslational modification of the apoproteins of the plant and algal biliproteins which occurs during their biosynthesis are also unknown. The present study provides a model system for one such biosynthetic pathway which entails the formation of a covalently bound heme protein precursor and its subsequent interconversion to the biologically active biliprotein.

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Photoinactivation of Peptide Transport in *Saccharomyces cerevisiae*†

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ABSTRACT: Oligopeptides and dipeptides are transported into *Saccharomyces cerevisiae* by a carrier-mediated system. In the dark, leucyl-*p*-nitroanilide (Leu-*p*-NA) and leucyl-leucyl-4-azido-2-nitrophenylalanine [Leu-Leu-Phe-($4N_3$,$2NO_2$)] are competitive inhibitors of peptide transport by *S. cerevisiae* cells. The photolysis of yeast cells in the presence of Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) at 350 nm results in an irreversible inactivation of peptide transport. Protection against this inactivation is afforded by an excess of trimethionine, a transported peptide. Photolysis with Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) does not affect amino acid or sugar transport, and cell viability is maintained throughout the irradiation procedure. A 5-min irradiation of *S. cerevisiae* with 2.4 μM Leu-*p*-NA or 15 μM Leu-Leu-Phe($4N_3$,$2NO_2$) causes 50% inhibition of trimethionine uptake. *p*-Nitroaniline, a possible hydrolysis product generated from Leu-*p*-NA by cellular peptidase activity, has no effect on peptide transport. An exogenous energy source is not required for photoinactivation. The results suggest that a component(s) of the peptide transport system of *S. cerevisiae* is irreversibly modified by photolysis with Leu-*p*-NA or Leu-Leu-Phe-($4N_3$,$2NO_2$) and provide the first example of the use of amino acid *p*-nitroanilides as photoaffinity labels.

Peptide transport has been studied in a variety of cells including bacteria (Payne & Gilvarg, 1978; Payne, 1980), fungi (Becker & Naider, 1980; Wolfinbarger, 1980), plants (Higgins & Payne, 1980), and cells of mammalian origin (Matthews & Payne, 1980). We have studied the uptake of peptides into *Saccharomyces cerevisiae* (Becker & Naider, 1980). Our results show that peptides are transported intact across the cell membrane of *S. cerevisiae*. The peptide transport system has highest affinity for peptides containing hydrophobic amino acids and is capable of taking up peptides composed of a variety of amino acids. Growth of cells in the presence of ammonium ion reduces the transport of peptides (Becker & Naider, 1977), as well as the transport of other nitrogen-containing small molecules in yeast (Grenson et al., 1970). Finally, in contrast to *Escherichia coli* which has separate di- and oligopeptide transport systems (Payne, 1980), *S. cerevisiae* appears to have one peptide transport system for all peptides utilized (Becker & Naider, 1980).

In a previous study (Parker et al., 1980), we showed that at pH 7.0 intact cells of *S. cerevisiae* 139 hydrolyzed leucyl-*p*-nitroanilide (Leu-*p*-NA)[1] and other aminoacyl-*p*-nitroanilides by an activity similar to that of aminopeptidase II, a well-characterized external peptidase in yeast (Frey & Rohm, 1979). Furthermore, we showed that at pH 5.5 Leu-*p*-NA was a competitive inhibitor of trimethionine transport in *S. cerevisiae* but does not affect amino acid transport. Transport inhibition was not related to hydrolysis of Leu-*p*-NA by aminopeptidase because at pH 5.5, the optimum for trimethionine transport, there was no aminopeptidase II activity. These results also provided evidence that peptides were not hydrolyzed by the extracellular aminopeptidase II prior to transport; if trimethionine were cleaved extracellularly followed by the transport of methionine, Leu-*p*-NA would not competitively inhibit. Other studies support the conclusion that peptides are not hydrolyzed extracellularly before transport into yeast: a peptide transport mutant retains peptidase activity (Marder et al., 1978), both transported and nontransported peptides are hydrolyzed by cell extracts (Naider et al., 1974), and amino acids do not compete for peptide transport in yeast (Marder et al., 1977).

A major goal of many transport studies is the isolation of components of the transport system. Chemical modification of transport proteins has been carried out for a variety of transport systems by affinity labeling techniques (Glover, 1977) to tag carriers. Photoaffinity reagents have been used to probe various transport systems because the highly reactive photolysis products generated in situ can react with virtually any amino acid side chain (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979). In the present study, incubation of cells with leucyl-*p*-nitroanilide or leucylleucyl-4-azido-2-nitrophenylalanine and irradiation with light of 350 nm led

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[1] Abbreviations: Leu-*p*-NA, leucyl-*p*-nitroanilide; Leu-Leu-Phe-($4N_3$,$2NO_2$), leucylleucyl-4-azido-2-nitrophenylalanine; Boc, *tert*-butyloxycarbonyl; OSu, hydroxysuccinimide ester.

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