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Biosynthesis of Phycobilins

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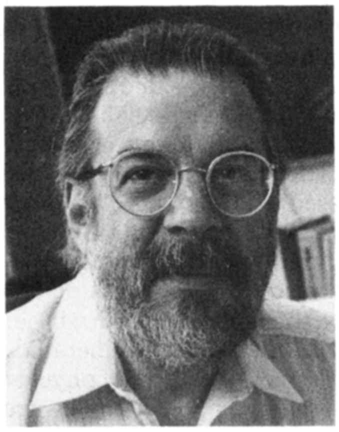
Contents



Samuel I. Beale received his B.S. (Chemistry, 1964), M.S. (Plant Science, 1966), and Ph.D. (Botany, 1970) degrees at the University of California, Los Angeles. He then carried out postdoctoral research at Brookhaven National Laboratory, University of California, Davis, the Marine Biology Laboratory at Woods Hole, MA, and The Rockefeller University. He has been at Brown University since 1979, where he is presently Professor of Biology in the Section of Biochemistry. Dr. Beale's interest in tetrapyrrole biosynthesis extends back to his graduate school research on the regulation of chlorophyll biosynthesis in algae. His principal research focus has been on the early steps of tetrapyrrole precursor biosynthesis in plants and microorganisms. He has also studied the biosynthesis of heme *a* and chlorophyll *b*, and the regulation of pigment synthesis by environmental and development factors, as well as the topic of this article.

I. Introduction

Phycobilins are open-chain tetrapyrroles that function as chromophores of light-harvesting chromoproteins in certain groups of photosynthetic organisms. In their functional state, the phycobilins are covalently linked to specific proteins, called phycobiliproteins. Two major classes of phycobiliproteins are phycocyanins and phycoerythrins, which are respectively colored blue and red. These pigments are largely responsible for the characteristic colors of the organisms which contain them. The different colors arise from slightly different phycobilin chromophores having different numbers of double bonds in conjugation. In cyanobacteria (formerly blue-green algae) and rhodophytes (red algae), phycobiliproteins, together with several "linker" polypeptides, form functional aggregates, called phycobilisomes, which are attached to the thylakoid membranes and mediate efficient light absorption and excitation transfer to the photosynthetic reaction centers. Cryptomonad algae also contain phycobiliproteins and utilize them for light harvesting, but the phycobiliproteins do not appear to be organized into phycobilisomes; instead, they are contained within the inner loculi of the thylakoid membranes. Phytochrome, an important photomorphogenetic pigment that is found in plants and some algae, is a biliprotein whose chromophore closely resembles the phycobilins. The phytochrome chromophore shares biosynthetic features with the phycobilins.

Because of their structural similarity to heme and heme-derived bilins, phycobilins have generally been considered to be products of the tetrapyrrole biosynthetic pathway. However, the specific enzymatic reactions leading to their formation were only poorly understood. Recent results from our laboratory and others have established the outlines of phycobilin

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biosynthesis and some of the characteristics of the enzymes that catalyze this process.

II. Phycobilin Structures

Phycobilins may be covalently linked to proteins by one or two thioether bonds (Figure 1). Those phycobilins that are attached to the protein through a single thioether bond at a reduced pyrrole ring can be liberated from the protein by heating to reflux temperature in methanol. The "methanolysis" products differ from the protein-bound pigments in that they contain an ethylidine group that is generated during cleavage of the thioether bond linking the pigment to the protein (Figure 2). The ethylidine double bond is conjugated with the chromophoric double bond system and causes the free chromophores to have somewhat different optical properties than the protein-bound forms. As will be discussed below, it is probable that ethylidine-containing free phycocyanobilin, phycoerythrobilin, and phytochromobilin are the biosynthetic precursors of the protein-bound forms of these pigments.

Historically, the first phycobilin whose structure was determined was methanolysis-liberated phycocyanobilin,[1,2] followed closely by that of phycoerythrobilin.[3,4] The analyses relied on ^{1}H NMR spectroscopy of the free pigments dissolved in deuterated organic solvents. The ethylidine groups of free phycobilins that were released from the proteins by overnight refluxing with methanol were reported to have the *E* configuration (Figure 2). Later, the *Z* isomers of phycocyanobilin and phycoerythrobilin were also detected after more gentle heating of phycocyanin and phycoerythrin with methanol.[5,6] As will be discussed below, the *Z* isomers of phycoerythrobilin and phycocyanobilin may be biosynthetic precursors of the *E* isomers.

The structures of several additional phycobilins have been described, bringing the present total number of distinct chromophores to eight, four of which are known to occur in both singly- and doubly-linked forms (Figure 1). Most of these pigments are not released from their proteins by methanolysis. Their structures were determined primarily by ^{1}H NMR spectroscopy of peptide-linked chromophores that were derived from purified phycobiliprotein subunits by proteolysis. The structures are named phycourobilin,[7] phycoviolobilin (also called phycobiliviolin and cryptoviolin),[8] bilin 584, and bilin 618.[9] Two recent additions are mesobiliverdin IXα and 15,16-dihydrobiliverdin IXα.[10] The pace of discovery of new phycobilins suggests that more structures remain to be uncovered as the pigments of more organisms are examined in detail.

In accord with their derivation from macrocyclic tetrapyrroles, phycobilins have *Z*-configured methine bridges as illustrated in Figure 1.[11] The phytochrome chromophore also has all *Z*-configured methine bridges in the red light absorbing form, but phototransformation to the far-red light absorbing form is accompanied by a *Z*-to-*E* isomerization of the 15-methine bridge, while the 5- and 10-methine bridges remain in the *Z* configuration.[12] It should be noted that the phycobilin structures illustrated in this article are drawn in the closed, or cyclohelical, conformation to facilitate visualization of their biosynthetic relationship to macrocyclic tetrapyrroles. Bilins that have *Z*-configured methine bridges tend to exist in cyclohelical conformation in solution.[13,14] However, spectroscopic and X-ray crystallographic studies indicate that in their functional state, phycobiliprotein chromophores are in an extended conformation,[13,15,16] as is the chromophore of phytochrome.[13]

III. In Vivo Studies on Phycobilin Biosynthesis

Before it became possible to detect biosynthetic reactions leading to phycobilins in vitro, intact organisms were examined for their ability to incorporate putative biosynthetic precursors into phycobiliprotein chromophores and for the effects of inhibitors that act specifically at certain biosynthetic steps. Results of these experiments established the biosynthetic origin of the carbon atoms of phycocyanobilin and indicated that heme and biliverdin are likely biosynthetic intermediates.

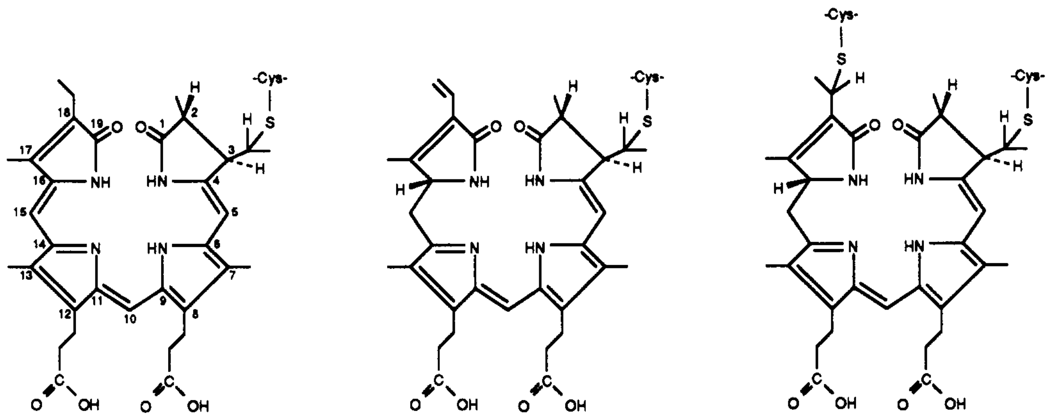
A. Early Precursors

The earliest universal tetrapyrrole precursor is the 5-carbon compound, δ-aminolevulinic acid (ALA). All of the carbon and nitrogen atoms in the porphyrin nucleus of tetrapyrroles are derived from ALA, and exogenous ^{14}C-ALA efficiently labels the phycobilins of cells growing in its presence.[17,18] ALA arises from condensation of glycine and succinyl-CoA in animals, yeasts, and some bacteria. In plants, algae, cyanobacteria, and other bacteria, ALA is formed by another route, utilizing the intact carbon skeleton of glutamic acid.[19]

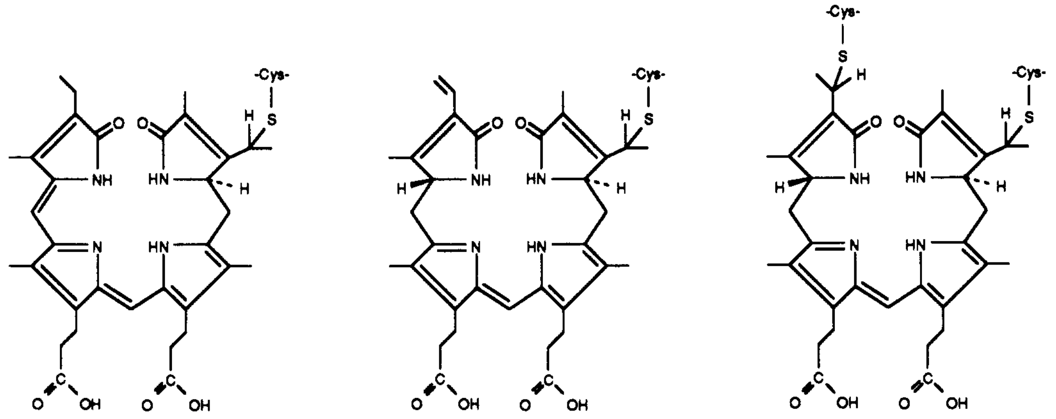
Levulinic acid is a competitive inhibitor of ALA dehydratase that causes ALA to accumulate in cells that are actively synthesizing tetrapyrroles.[20] Most or all of the ALA that accumulates when cyanobacteria and red algae are grown in the presence of levulinic acid is derived from glutamate.[21-24] This is true even in mutant cells of the unicellular red algae *Cyanidium caldarium* that have lost the ability to form photosynthetic pigments.[25] Weinstein and Beale[26] also found that in *C. caldarium*, all cellular hemes, including mitochondrial heme *a*, are formed from ALA that is synthesized from glutamate. More recently, in vitro conversion of glutamate to ALA has been measured in extracts of *C. caldarium*[27,28] and several cyanobacteria.[29,30] It thus appears that, in two of the three groups of phycobilin-forming organisms, cyanobacteria and red algae, all tetrapyrroles are derived from glutamate, and therefore the first step of phycobilin biosynthesis in these organisms is ALA formation from glutamate. The third group of phycobilin-containing organisms, the cryptophytes, have not been examined for their route of ALA biosynthesis.

B. Heme as a Precursor

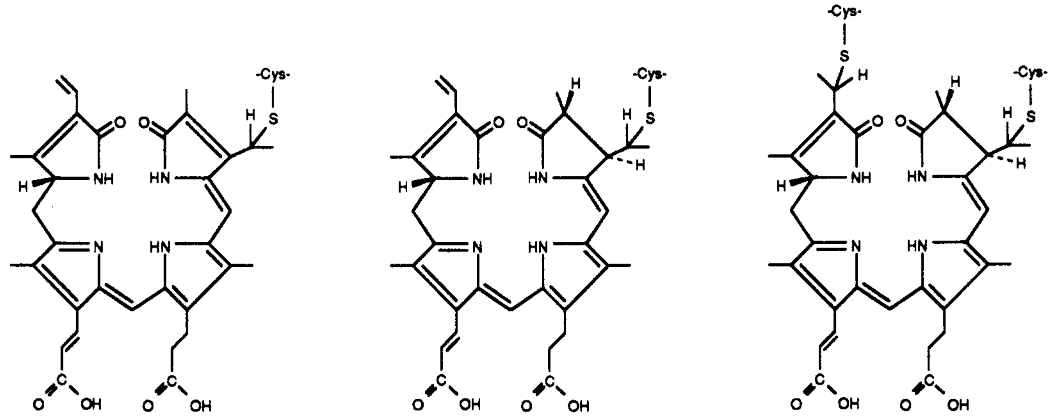
The participation of heme in phycobilin formation is suggested by similarities of the phycobilins to the tetrapyrrole macrocycle ring-opening reaction products appearing in animal heme catabolism. Indirect evidence supporting the precursor status of heme was obtained by Beale and Chen.[31] *N*-Methyl mesoporphyrin IX is a specific inhibitor of enzymatic iron insertion into protoporphyrin IX and therefore blocks heme formation.[32] Administration of *N*-methyl mesoporphyrin IX to growing *C. caldarium* cells caused inhibition of phycocyanin, but not chlorophyll formation. This result suggested that heme is a direct



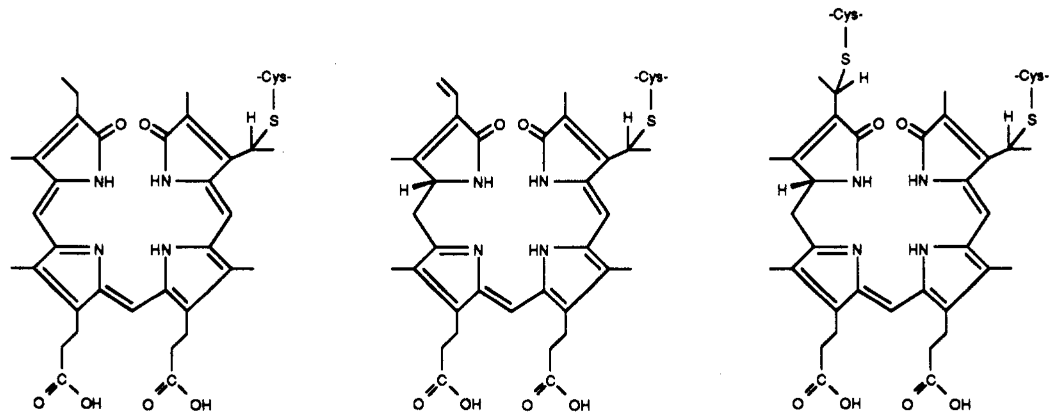
Cys-Phycocyanobilin Cys-Phycoerythrobilin DiCys-Phycoerythrobilin



Cys-Phycoviolobilin Cys-Phycourobilin DiCys-Phycourobilin



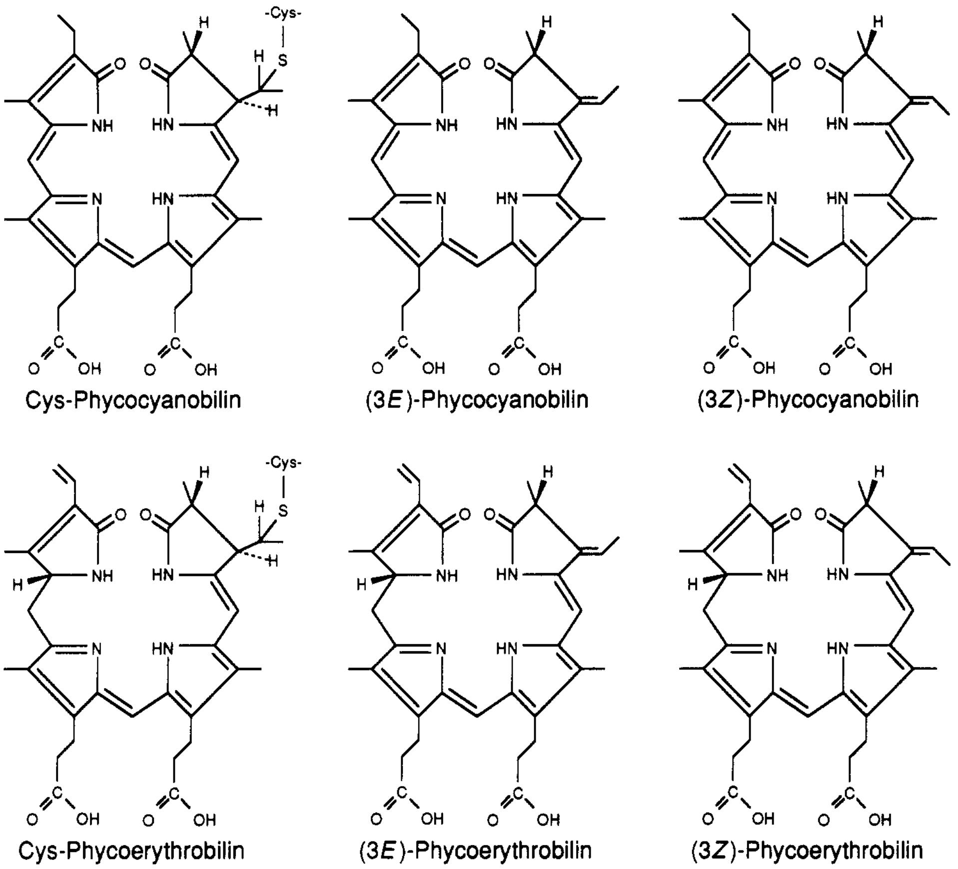
Cys-Bilin 618 Cys-Bilin 584 DiCys-Bilin 584



Cys-Mesobiliverdin IXα Cys-15,16-Dihydrobiliverdin IXα DiCys-15,16-Dihydrobiliverdin IXα

Figure 1. Structures of protein-linked phycobilins. The conventional bilin numbering system is shown for phycocyanobilin.

precursor of phycobilins. The experiment was carried out in the dark using a mutant strain which normally forms both pigments in the dark. In the light, synthesis of both phycocyanobilin and chlorophyll was inhibited by *N*-methyl protoporphyrin IX in wild-type cells,[33] but the effect on chlorophyll could have been an indirect



Cys-Phycocyanobilin (3*E*)-Phycocyanobilin (3*Z*)-Phycocyanobilin

Cys-Phycoerythrobilin (3*E*)-Phycoerythrobilin (3*Z*)-Phycoerythrobilin

Figure 2. Free and protein-bound phycocyanobilin and phycoerythrobilin. Chromophores that are linked to the proteins by a single thioether bond adjacent to a reduced pyrrole ring are released by heating in methanol, to yield primarily the (3*E*)-ethylidine isomers, plus smaller amounts of the (3*Z*) isomers.

one caused by phototoxic effects of the administered inhibitor, which is a photodynamically active porphyrin.

Direct evidence for the precursor status of heme was reported by Brown et al.[17] and Schuster et al.,[34] who showed that exogenous ^{14}C-heme could contribute label to phycocyanobilin in greening *C. caldarium* cells. The specificity of heme incorporation was indicated by the fact that unlabeled chlorophyll was formed simultaneously with the labeled phycocyanobilin when greening cells were incubated with ^{14}C-heme. Further evidence supporting a role for heme was provided by the observation that nonradioactive heme was able to decrease the incorporation of ^{14}C-labeled ALA into phycocyanobilin.[17] Earlier studies had reported low but significant levels of ^{14}C-heme incorporation into phycocyanobilin by cells of *Anacystis*, a cyanobacterium.[35] However, these results could not be repeated by Brown et al.,[36] possibly due to the impermeability of the cells to heme.

C. Biliverdin as a Precursor

Biliverdin was reported to accumulate in the culture medium of *C. caldarium* cells that were grown in the dark with exogenous ALA.[37] Although this result was interpreted as evidence that biliverdin is a precursor of phycocyanobilin, the biliverdin could have accumulated as a result of degradation of excess heme that might have been formed as a result of ALA administration.

Beale and Cornejo[38] found that the phycocyanin chromophore became labeled when purified ^{14}C-biliverdin IXα was administered to *C. caldarium* cells growing in the dark in the presence of *N*-methyl mesoporphyrin IX, which was added to block endogenous heme formation. The strain of cells used in these experiments was capable of forming phycocyanin in the dark, and dark growth was used to eliminate possible phototoxic effects of administered *N*-methyl mesoporphyrin or biliverdin. Cellular protoheme remained unlabeled during the incubations with ^{14}C-biliverdin, indicating that the incorporation of label into phycocyanobilin was direct, rather than via degradation of the administered labeled compound and subsequent reutilization of the ^{14}C. The ability of exogenous ^{14}C-biliverdin to label the phycocyanin chromophore in vivo was confirmed.[39,40]

IV. Analogous Enzyme Reactions in Heme Metabolism by Animals

In animal cells, heme that is released upon the degradation of hemoglobin, myoglobin, and cytochrome P-450 is converted to biliverdin. Many animal species excrete biliverdin, but mammals and some fish[41] first reduce the biliverdin to bilirubin before conjugation and excretion. Although the heme metabolism that occurs in animals is primarily catabolic, as contrasted to the biosynthetic heme metabolism in phycobilin formation, the catabolic reactions have some similarities to the biosynthetic reactions and therefore can serve as mechanistic models for the latter.

A. Microsomal Heme Oxygenase

Conversion of the aromatic porphyrin macrocycle to the open-chain bilin structure is catalyzed by heme

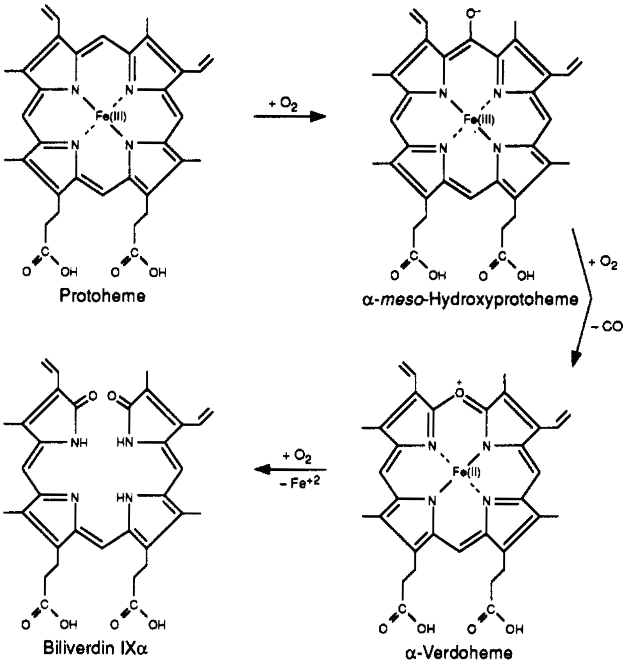


Figure 3. Pathway for conversion of heme to biliverdin, showing the structures of the principal intermediates.

oxygenase in animal cells that carry out heme degradation. Animal heme oxygenase is a microsomal monooxygenase that requires O_2 and NADPH as cosubstrates. Tenhunen et al.[42,43] established that the conversion of each molecule of heme to biliverdin requires three molecules of O_2 and at least three of NADPH, the excised α-meso carbon of heme is released as CO, and label from $^{18}O_2$ is incorporated into both products, biliverdin and CO, but label from $H_2{}^{18}O$ is not. Isotope studies, in both intact cells[44,45] and purified, reconstituted systems,[46] established that each of the lactam oxygen atoms appearing in biliverdin is derived from a different O_2 molecule. Oxygenated ferrous heme, α-*meso*-hydroxyheme, and a species absorbing at 688 nm that has been tentatively identified as α-verdoheme have been detected as intermediate reaction products (Figure 3).[47-50]

Figures 4–6 show proposed reaction mechanisms for each of the three oxygenation steps catalyzed by heme oxygenase. Initially, Fe(III)-protoheme binds to heme oxygenase, where it is reduced to Fe(II)–protoheme by an electron provided by NADPH via NADPH–cytochrome *c* reductase. The Fe(II)–heme binds an O_2 molecule, and then the complex undergoes an intramolecular electron transfer from the Fe atom to the O_2, to produce the Fe(III)–superoxide anion radical. The radical then attacks the macrocycle at the α-meso position to form the α-*meso*-hydroperoxide with an unpaired electron on the macrocycle. Loss of H_2O and injection of another electron produces Fe(III)–α-*meso*-hydroxyprotoheme.[48] Although hydroxylation at the α-meso bridge position is required for eventual opening of the macrocycle at this position, the hydroxylation site is not the sole determinant of the end product isomer: of the four possible *meso*-hydroxyprotoheme isomers (chemically synthesized), only the α-meso isomer was converted to the corresponding biliverdin isomer by bovine liver heme oxygenase.[50] The other three *meso*-hydroxyprotoheme isomers were poor substrates for further reaction.

After α-*meso*-hydroxyprotoheme is formed, an electron is transferred from the macrocycle to the Fe(III), to form Fe(II)–α-*meso*-hydroxyprotoheme with an unpaired electron on the macrocycle. A second O_2 molecule binds at the Fe(II), the superoxide anion radical is formed via internal electron transfer from Fe, as before, and then the superoxide is transferred to the macrocycle. The hydroperoxide oxidatively reacts at the α position of the adjacent pyrrole ring and CO is excised, yielding the 688-nm-absorbing compound (probably α-verdoheme).[49] During CO excision and O-bridge formation, the Fe(III) atom remains coordinated with the pyrrole N atoms, keeping the two pyrrole α positions that were adjacent to the α-methine carbon in close proximity to facilitate bridging by the O atom that is derived from the second O_2 molecule. After the O bridge is formed, the Fe is reduced to form Fe(II)–α-verdoheme. The third molecule of O_2 is added by an analogous process of superoxide anion radical formation and transfer, and the superoxide is then reduced to form the hemiketal form of ferric biliverdin. Finally, the Fe atom is reduced and both biliverdin IXα and Fe^{2+} are released.

It is noteworthy that all three oxygenation steps, as well as all other steps of the heme oxygenase reaction, are catalyzed by a single, small enzyme, which probably binds a single tetrapyrrole molecule throughout the reaction sequence. In the proposed reaction sequence, the details of the three oxygenation steps are very similar. In all three cases, the common mechanism of O_2 activation, internal electron transfer from Fe(II)–heme to form the Fe(III)–superoxide anion radical, can occur at a single catalytic site. The reactive oxygen radical species are successively transferred to the progressively more oxidized "top" of the tetrapyrrole, eventually causing CO excision and biliverdin formation. In the model, a total of seven electrons are required for the conversion of Fe(III)–heme to biliverdin plus Fe^{2+}, a number that is consistent with the requirement for "at least three" molecules of NADPH.[42,43]

Key features of the microsomal heme oxygenase reaction are observed in phycobilin formation in vivo. When intact *C. caldarium* cells form phycocyanin in the presence of [^{14}C-5]ALA, ^{14}CO and ^{14}C-phycocyanobilin are formed in equimolar amounts.[18] Also, each of the lactam oxygen atoms of phycocyanobilin is derived from a different O_2 molecule.[36,51]

Purified microsomal heme oxygenase from bovine spleen microsomes has a molecular weight of 31 000 and a K_m for heme of 1 μM.[52] Reconstitution of activity required the presence of a 78 000 molecular weight NADPH–cytochrome *c* reductase (also called NADPH–cytochrome P-450 reductase), which serves to mediate transfer of reducing equivalents from NADPH to oxygenase-bound heme. Strong interaction between heme oxygenase and NADPH–cytochrome *c* reductase was observed in vitro.[53] As would be expected for an enzyme that has essential histidine residues, heme oxygenase is inactivated by diethyl pyrocarbonate.[54] Microsomal heme oxygenase is also inhibited by certain metalloporphyrin heme analogs such as Sn–, Mn–, Zn–, and Co–protoporphyrin IX.[52] Biliverdin has also been reported to act as a competitive inhibitor of heme oxygenase.[55]

Two isoenzymes of heme oxygenase have been detected in mammals[56,57] and birds.[58] The two forms

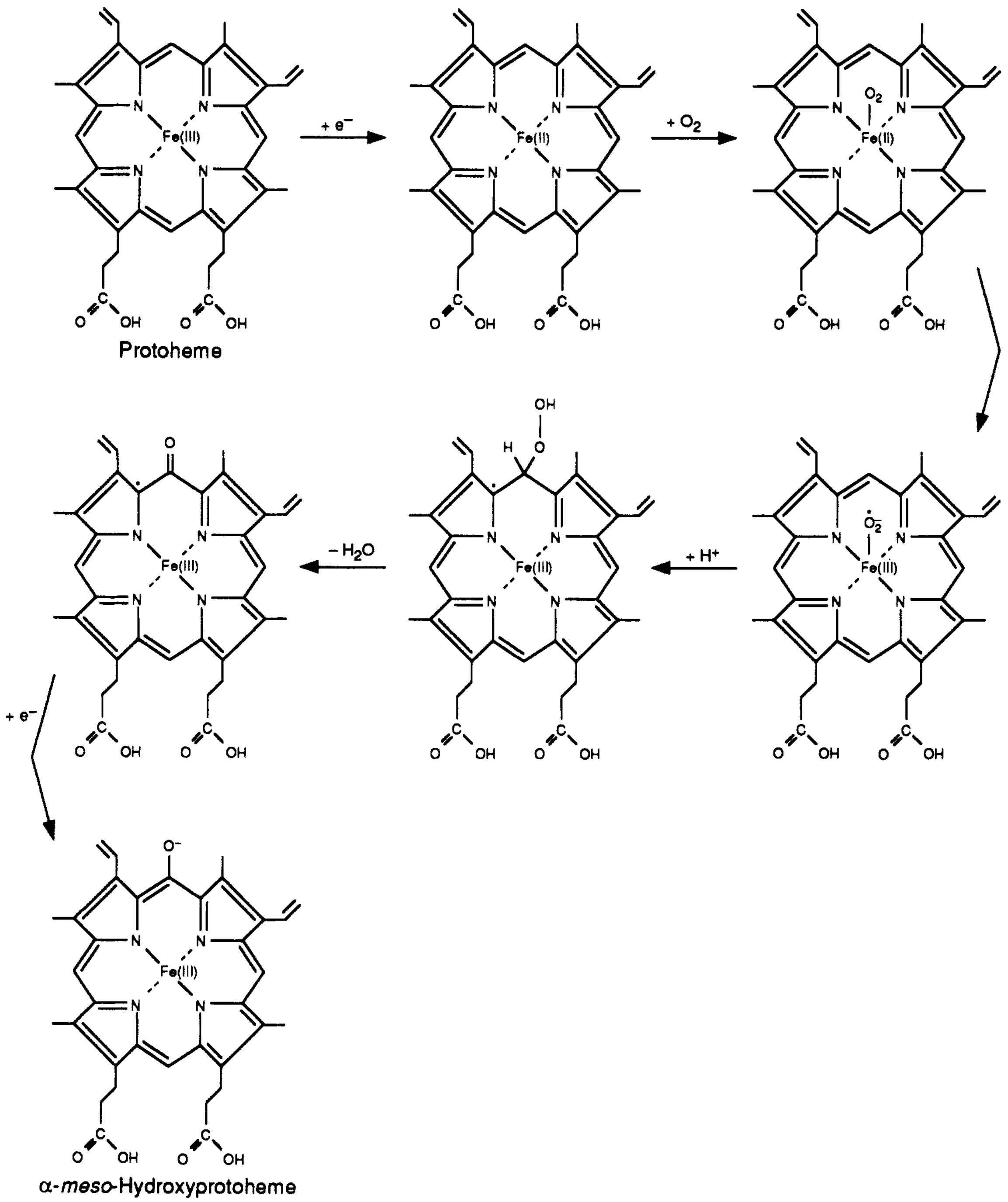


Figure 4. Proposed mechanism for the first oxygenation step of the heme oxygenase reaction.

are encoded by different genes[59,60] and are differentially inducible in different tissues.[61] Even though the two forms are immunologically distinguishable,[58,59,61] they share extensive conserved regions[59,62] and have similar substrate specificities.[63] A conserved histidine residue has been identified by site-specific mutagenesis as essential for enzyme activity.[64]

In the spleen, hemoglobin degradation is the major source of substrate heme for heme oxygenase. Heme in native hemoglobin is not degradable by the enzyme, but the heme in separated α and β chains of hemoglobin is degraded by heme oxygenase.[63] In the liver, the heme in cytochrome P-420, a partially-degraded form of cytochrome P-450, can apparently be attacked by heme oxygenase, and the heme in some forms of native cytochrome P-450 may also be directly degraded by the enzyme.[65] Also, heme *c* that is derived by proteolytic digestion of cytochrome *c* is converted to biliverdin *c* by heme oxygenase in vitro,[66] but intact cytochrome *c* is not degraded by heme oxygenase.[61]

Microsomal NADPH-cytochrome *c* reductase by itself can catalyze heme breakdown in vitro,[54,67,68] as can a membrane fraction from heart mitochrondria,[69] but these reactions involve H_2O_2 generation and do not produce biliverdin IXα and CO specifically and stoichiometrically as does heme oxygenase.

B. Mammalian Biliverdin Reductase

All but one of the known phycobilins are chemically more reduced than biliverdin by two or four electrons. Bilirubin, the major bilin excreted by mammals, is more reduced than biliverdin by two electrons. The biliverdin reduction step that occurs in mammalian heme catabolism may have mechanistic relevance to phycobilin synthesis. Mammalian biliverdin reductase specifically

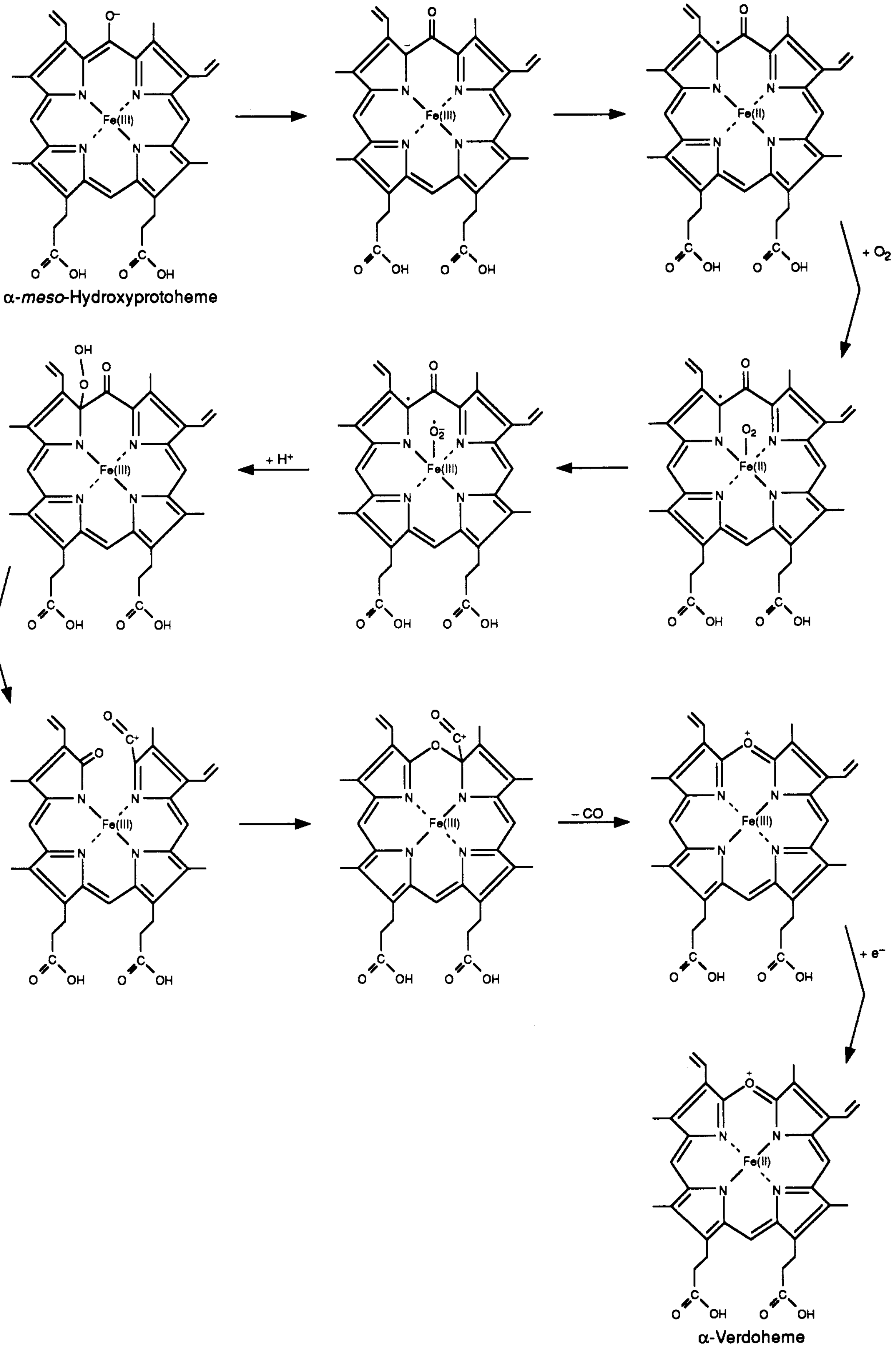


Figure 5. Proposed mechanism for the second oxygenation step of the heme oxygenase reaction.

reduces the C-10 methene bridge of biliverdin to methylene. Phycobilins with a reduced 10-bridge are unknown, but phycobilins may be reduced at the C-5 bridge, the C-15 bridge, or both (Figure 1).

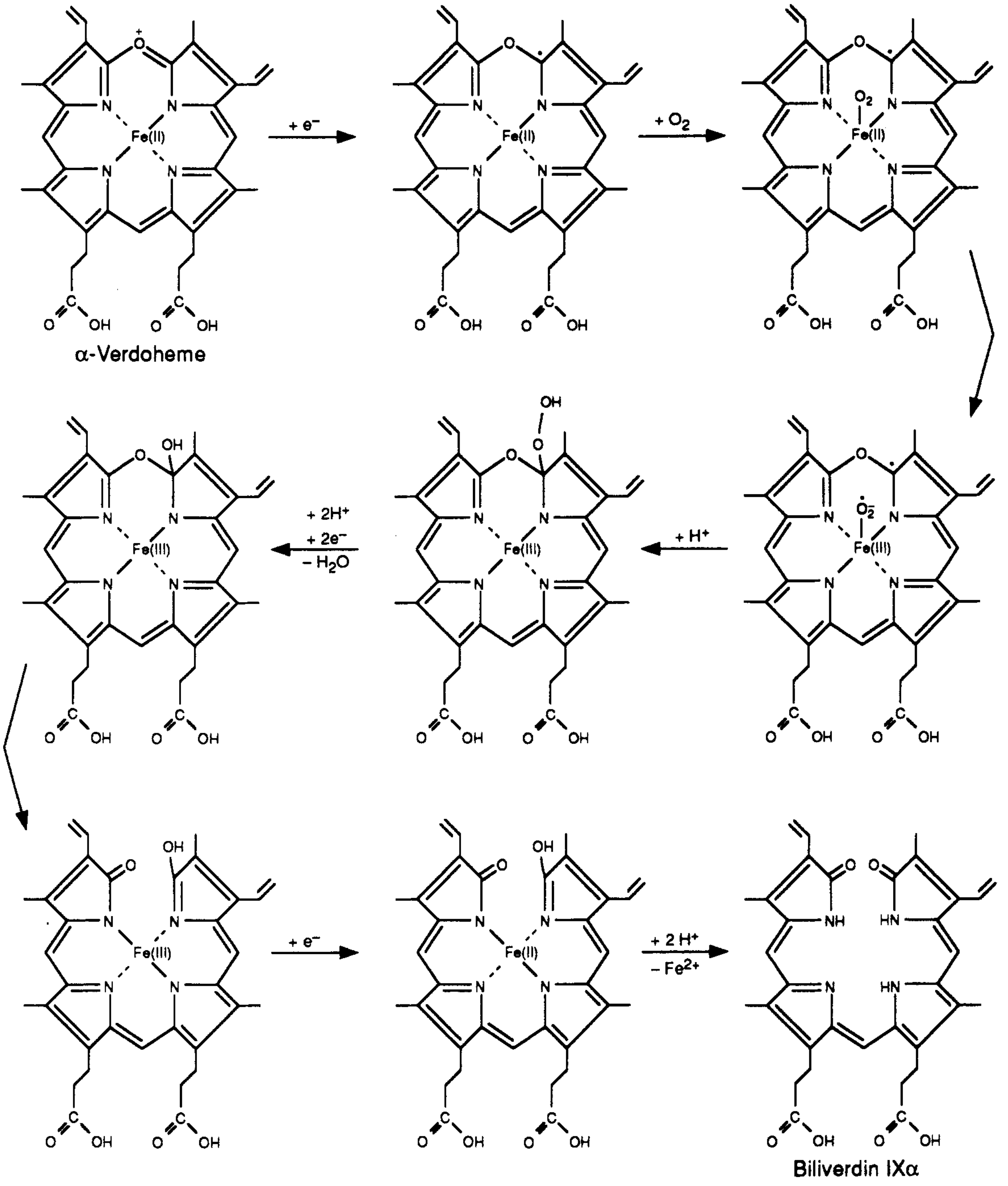


Figure 6. Proposed mechanism for the third oxygenation step of the heme oxygenase reaction.

In contrast to microsomal heme oxygenase, mammalian biliverdin reductase is a soluble cytoplasmic enzyme.[70,71] The purified enzyme from rat liver has a molecular mass of 35 000 Da, prefers NADPH over NADH as the reductant (K_m = 3.0 μM for biliverdin with NADPH as reductant, 270 μM with NADH), has a pH optimum of 8.7 with NADPH, and is inhibited by biliverdin concentrations above 5 μM.[71] Biliverdin reductase from eel liver is larger (molecular weight 67 000), has a higher affinity for biliverdin (K_m = 0.6 μM), and has a lower pH optimum of 5.3.[41] Three isotypes of biliverdin reductase have been reported in rat liver,[72] but all three are apparently encoded by a single gene.[73] The different forms of the rat liver enzyme have varying degrees of activity with different nonphysiological biliverdin isomers,[74,75] and mesobiliverdin IXα is also reduced to mesobilirubin IXα by the rat liver enzyme.[76] Bovine spleen biliverdin reductase forms an association with heme oxygenase in vitro.[53]

V. *In Vitro* Studies on Phycobilin Biosynthesis

By 1983, the involvement of heme and biliverdin in phycobilin biosynthesis had been established by the in vivo experiments described above. Cell-free extracts from phycobilin-forming organisms that are capable of catalyzing biliverdin formation from heme and phycocyanobilin formation from biliverdin were first described in 1984.[5,77] These reports, and the results of subsequent experiments that have partially characterized these reactions, are described below.

A. Transformation of Heme to Biliverdin by Extract of *Cyanidium caldarium*

Beale and Cornejo[77] detected the heme oxygenase reaction in extracts of *C. caldarium*. Like the animal system, the unfractionated algal heme oxygenase system required reduced pyridine nucleotide (NADPH was about twice as effective as NADH) as well as molecular

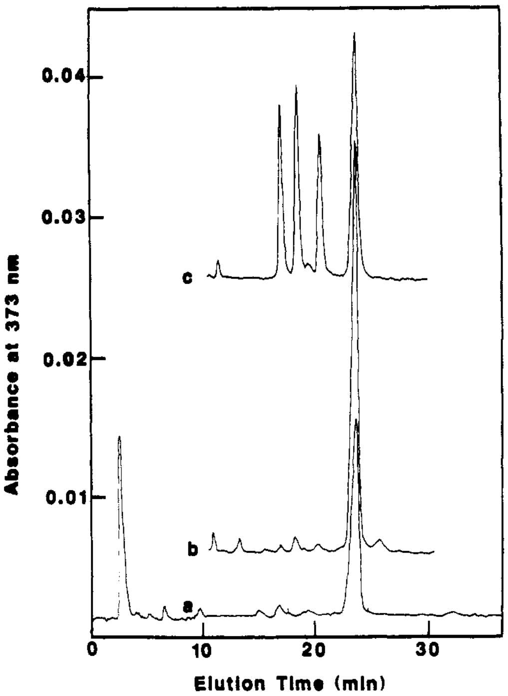


Figure 7. (a) HPLC elution profile of the algal heme oxygenase incubation product with protohemin as the substrate. (b) Relevant portion of the HPLC elution profile of mixture of the incubation product plus 0.70 nmol of standard biliverdin IXα. (c) Relevant portion of the HPLC elution profile of a mixture of the four biliverdin IX isomers generated by coupled oxidation of protoheme in the presence of ascorbate and pyridine. Reprinted from ref 78. Copyright 1988 *Journal of Biological Chemistry.*

oxygen. Ascorbate and other moderately strong reductants stimulated the reaction in unfractionated cell extracts and were required after removal of low-molecular-weight materials from the enzyme system by gel filtration or dialysis. It is possible that these strong reductants function to reduce the Fe of heme to the ferrous state, rather than serving as enzyme substrates.[78] Like the microsomal enzyme,[79] algal heme oxygenase is powerfully inhibited by Sn–protoporphyrin IX. In contrast, heme degradation via nonspecific reactions, catalyzed by hemoproteins such as myoglobin, is not inhibited by Sn–protoporphyrin.[77]

1. Identification of the Reaction Product as Biliverdin IXα

Originally, it was necessary to utilize mesoheme in place of the physiological substrate protoheme. The advantage of mesoheme is that the reaction product, mesobiliverdin, is not a natural product and can be distinguished from residual biliverdin that may be present in cell extracts. Moreover, because mesobiliverdin is not a substrate for phycobilin formation, it is more stable than biliverdin in the cell extract, and thus is more likely to accumulate to detectable levels during the incubation. After the initial detection of heme oxygenase activity using mesoheme as a substrate, conversion of protoheme to biliverdin was also detected with optimized incubation conditions and partially purified enzymes.[78]

The products of the reaction with mesoheme and protoheme were identified as the IXα isomers of mesobiliverdin and biliverdin (Figure 7), respectively.[77,78] In contrast to the exclusive production of IXα isomers by the enzyme reaction, biliverdin produced by chemical attack on hemes by ascorbate in aqueous pyridine is a mixture of all four ring-opening isomers,[77,78] and coupled oxidation of myoglobin-bound heme also produces an isomer mixture.[77]

2. Enzymes of the Algal Heme Oxygenase System

The algal heme oxygenase system differs from the animal cell-derived microsomal system in that it is soluble, with virtually all of the activity appearing in the high-speed supernatant fraction.[77] This finding is consistent with the fact that the reaction is thought to occur in the plastids, and is also presumed to occur in prokaryotic cyanobacteria. Neither plastids nor prokaryotes have microsomes.

In contrast to the two-component microsomal heme oxygenase system, the algal heme oxygenase system can be separated into three required protein components: a ferredoxin-dependent NADPH–cytochrome *c* reductase; a small Fe/S protein that appears to be ferredoxin; and a heme-binding reductase.[78] The reconstituted heme oxygenase system required all three protein components for activity. The ferredoxin-dependent NADPH–cytochrome *c* reductase could be replaced by spinach ferredoxin–NADP$^+$ reductase, and the ferredoxin could be replaced by ferredoxin derived from spinach or the red alga, *Porphyra umbilicalis*.[78,80]

A mechanistic question was whether the sole role of NADPH in the reaction was to serve as an electron source for ferredoxin reduction, or whether NADPH directly donates one or more electrons to heme oxygenase. To answer this question, heme oxygenase activity was assayed in incubation mixtures containing *C. caldarium* heme oxygenase without added NADPH but supplemented with a light-driven ferredoxin reduction system derived from partially purified photosystem I of spinach leaves. In this mixed reconstitution assay, heme oxygenase activity was light dependent.[80] In the dark, no activity was detected unless NADPH and ferredoxin–NADP$^+$ reductase were added to the incubation mixtures. It can be concluded from these results that the sole essential role of NADPH and ferredoxin–NADP$^+$ reductase in the algal heme oxygenase system is to reduce ferredoxin and that ferredoxin is the direct electron source for heme oxygenase.

It is likely that both photosystem I- and NADPH-derived electrons for ferredoxin reduction are important for phycobilin synthesis in vivo. In the light, ferredoxin is presumably reduced by the action of photosystem I. However, since the *C. caldarium* strain used for these studies is capable of synthesizing phycobilins in the dark as well as in the light,[31] the cells must be capable of reducing ferredoxin in the dark, using ferredoxin–NADP$^+$ reductase to transfer electrons from NADPH.

Still unclarified is the role of ascorbate in the algal heme oxygenase reaction. Ascorbate, or some other fairly strong reductant such as isoascorbate or phenylenediamine, is required in addition to NADPH and ferredoxin–NADP$^+$ reductase, for activity in enzyme preparations from which the naturally-occurring low molecular weight components have been removed.[78] The requirement for the reductant could not be tested in the heme oxygenase reaction when NADPH and ferredoxin–NADP$^+$ reductase were replaced by spinach photosystem I-catalyzed light-driven ferredoxin reduction, because ascorbate is needed as the electron

Table I. Characterization of Bilins by HPLC Elution Times and Visible Absorption Maxima[a]

pigment	source	HPLC elution time, min	absorption maxima, nm	
			our data	literature
biliverdin IXα	commercial	12.4[6]	377, 696[6]	377, 696[85]
(3*Z*)-phycocyanobilin	*C. caldarium* methanolysis	17.1[6]	369, 686[6]	
(3*Z*)-phycocyanobilin	enzyme incubation	17.1[6]	369, 686[6]	
(3*Z*)-phycocyanobilin	chemical synthesis			368, 685[86,b]
(3*Z*)-phycoerythrobilin	*P. cruentum* methanolysis	21.0[6]	327, 591[6]	
(3*Z*)-phycoerythrobilin	enzyme incubation	21.0[6]	327, 591[6]	
(3*E*)-phytochromobilin	*P. cruentum* methanolysis	23.2	386, 700[84]	
(3*E*)-phytochromobilin	chemical synthesis			386, 708[86,b]
(3*Z*)-phytochromobilin	chemical synthesis			382, 702[86,b]
15,16-dihydrobiliverdin IXα	enzyme incubation	28.2[84]	335, 560[87]	
(3*E*)-phycocyanobilin	*C. caldarium* methanolysis	33.8[6]	375, 692[6]	374, 690[1]
(3*E*)-phycocyanobilin	enzyme incubation	33.8[6]	375, 692[6]	
(3*E*)-phycoerythrobilin	*P. cruentum* methanolysis	41.0[6]	329, 592[a]	328, 590[88]

[a] Reverse-phase HPLC was on a 4.6-mm-diameter × 25-cm-long column of octadecylsilane-coated 5-μm-diameter spherical silica particles (Altex). The elution solvent was ethanol/acetone/water/acetic acid (48:34:17:1, v/v) flowing at 4.0 ml/min. Visible absorption spectra of the HPLC-purified pigments were recorded in methanol/36% (w/v) aqueous HCl (49:1, v/v). Adapted from Cornejo et al.[84]
[b] Data are for the dimethyl ester.

source for photosystem I. The fact that isoascorbate is equally effective as ascorbate suggests that the interaction of the reductant with the heme oxygenase system is chemical rather than enzymatic. We have pointed to the high natural concentrations of ascorbate in chloroplasts and suggested that plastids may normally employ ascorbate for maintaining the Fe of heme in the ferrous state, instead of having a specific ferric heme reducing function of heme oxygenase.[78] It will be difficult to test for this role for ascorbate directly by determining if there is still an ascorbate requirement when ferrous heme is used as the substrate, because the heme oxygenase reaction requires O_2, and ferrous heme is unstable in this environment in the absence of a strong reductant such as ascorbate. However, it may be possible to determine the role of ascorbate indirectly by substituting Co–protoporphyrin for heme in the reaction. Co–protoporphyrin is a substrate for some microsomal heme oxygenases under some conditions.[81] To function as a heme oxygenase substrate, the oxidation state of Co in Co–protoporphyrin must be Co(II).[82] Ascorbate can not reduce Co(III) to Co(II), and a stronger reductant, such as dithionite, is required.[82] If the algal heme oxygenase system can accept Co–protoporphyrin as a substrate in the presence of dithionite, but not when ascorbate is substituted for dithionite, then it would be likely that the role of the reductant is to keep the metal reduced.

Another possible role for ascorbate would be to nonenzymatically reduce the Fe in the penultimate reaction product, ferric–biliverdin. In the microsomal heme oxygenase system, reduction of ferric–biliverdin is necessary for release of biliverdin from the enzyme and to allow reaction with a new heme molecule to then proceed. It is possible that the algal enzyme, which normally functions in the presence of ascorbate in the plastids, is incapable of enzymatically reducing ferric–biliverdin and relies on ascorbate for this function.

The heme-binding component of the algal heme oxygenase system has an apparent native molecular weight of approximately 38 000 and is resistant to inactivation by *p*-(hydroxymercuri)benzoate.[78] The enzyme is inactivated by diethyl pyrocarbonate, and the inactivation is blocked by heme. In the reconstituted heme oxygenase system, the heme-binding component was the rate limiting one, e.g., addition of this component to an unfractionated cell extract increased the yield of biliverdin, whereas addition of the ferredoxin or ferredoxin–NADP$^+$ reductase components did not increase the biliverdin yield.

B. Transformation of Biliverdin to Phycocyanobilin by Extract of *Cyanidium caldarium*

Because nearly all phycobilins that have so far been described contain at least two more hydrogen atoms than biliverdin, some form of biochemical reduction is required for the transformation of biliverdin to these phycobilins. Although early attempts at detection of biliverdin reduction activity in algal extracts were not successful,[83] Beale and Cornejo[5] measured enzymatic conversion of biliverdin to free phycocyanobilin in cell-free extracts of *C. caldarium*. In addition to biliverdin IXα, the reaction required a reduced pyridine nucleotide, NADPH being more effective than NADH. Activity was retained in the high-speed supernatant fraction and eluted with the protein fraction on gel filtration.

1. Identification of the Final Reaction Product as (3E)-Phycocyanobilin

Identification of the principal product of biliverdin reduction by unfractionated *C. caldarium* extract as (3*E*)-phycocyanobilin was by comparative absorption spectroscopy[1,6,84–88] and reverse-phase HPLC (Table I). The absorption maxima and HPLC elution time were identical to those of authentic (3*E*)-phycocyanobilin, which is the principal product obtained from methanolysis of phycocyanin.[1] The incubation product was further identified by chemical transformation to mesobiliverdin.[5,37]

2. Identification of (3Z)-Phycocyanobilin as an Intermediate

Incubation products of the reaction included both the (3*Z*)- and (3*E*)-ethylidine isomers of phycocyanobilin.[5] The identity of (3*Z*)-phycocyanobilin was established by comparative absorption spectroscopy and reverse-phase HPLC (Table I) and confirmed by ^1H NMR.[89] Interestingly, both ethylidine isomers of phycocyanobilin are also formed upon methanolytic cleavage of the phycocyanin chromophore from the protein moiety,[90] but the *Z* isomer, being less stable,[86] isomerizes to the E isomer at the elevated temperatures employed for methanolysis, and the equilibrium isomer ratio strongly favors the E form. The Z- and E-ethylidine isomers of phycocyanobilin could be interconverted by heating in acetic acid–methanol mixtures.[5]

At early incubation times, the Z isomer was the predominant reaction product.[5] The time courses for the appearance of the two isomers suggests a precursor–product relationship between the Z and E forms. Preliminary evidence indicates that enzymatic isomerization is catalyzed by cell extract in the presence of reduced glutathione.[89] After removal of low-molecular-weight material from unpurified cell extract by Sephadex G-25 gel filtration, (3Z)-phycocyanobilin was the major product formed enzymatically. Addition of GSH to incubations containing gel-filtered cell extract restored the ability to form (3E)-phycocyanobilin. Moreover, purified (3Z)-phycocyanobilin was converted to (3E)-phycocyanobilin by the cell extract, and this conversion was greatly stimulated by GSH. The reverse conversion was not detected. In the absence of enzyme, GSH did not catalyze the conversion. These results indicate that the cell extract contains GSH-dependent enzyme activity that isomerizes the ethylidine group of (3Z)-phycocyanobilin to the E configuration.

This reaction falls into the category of cis–trans isomerizations that do not involve migration of the double bond.[91] Enzymes comprising this class of isomerases require sulfhydryl groups for activity. One subclass of these enzymes uses integral enzyme sulfhydryls, and a second subclass uses sulfhydryls supplied by a cofactor.[91] Several members of this enzyme subclass, including maleate isomerase and several maleyl isomerases, specifically require GSH for activity.[91,92] It is not known whether GSH or some other low molecular weight cofactor is the natural in vivo cofactor that is removed from *C. caldarium* cell extract during purification of the protein fraction which catalyzes the ethylidine isomerization.

The existence of the (3Z)-phycobilins may be rationalized by proposing that the 2,3-reductase reaction that forms the reduced pyrrole ring uses, as substrate, a bilin that has a 3-vinyl group and produces, as the immediate product, a 3-vinyl-2,3-dihydrobilin. Gossauer et al.[93] have shown that synthetic 3-vinyl-2,3-dihydrobilins spontaneously isomerize to (3Z)-3-ethylidene-2,3-dihydrobilins. Thus, enzymatic pyrrole reduction of biliverdin or another 3-vinylbilin would initially yield the unstable 3-vinyl-2,3-dihydrobilin, which would nonenzymatically isomerize to the (3Z)-3-ethylidene-2,3-dihydrobilin. However, if only the more stable (3E)-phycobilins are acceptable substrates for chromophore ligation to the apoproteins, there would be a need for a Z–E isomerase to transform the products of pyrrole ring reduction to the ligase substrates. The existence of a 3-vinyl-2,3-dihydrobilin intermediate is consistent with the production of α-hydroxymesobiliverdin, along with biliverdin, when ALA was administered to *C. caldarium* cells in the dark.[94] Although the authors of the report suggested that the α-hydroxymesobiliverdin IXα might arise as a degradation product of phycocyanobilin, another interpretation is suggested: If the pyrrole reduction step of phycocyanobilin formation acts on a precursor that contains a 3-vinyl group, the resulting 3-vinyl-2,3-dihydrobilin product would spontaneously isomerize to the (3Z)-3-ethylidine-2,3-dihydrobilin.[93] Under some conditions, an alternative fate of the vinyl-containing intermediate might be for water to add across the reactive vinyl group, forming α-hydroxymesobiliverdin.

3. Identification of (3Z)-Phycoerythrobilin as an Intermediate

In the course of fractionation and purification of the proteins in the *C. caldarium* extract that catalyze the transformation of biliverdin to phycocyanobilin, it was observed that incubation produced other pigmented products in addition to phycocyanobilin. One of these products was identified as (3Z)-phycoerythrobilin by comparative absorption spectroscopy and HPLC elution (Table I), and the identification was confirmed by ^{1}H NMR.[6] The generation of (3Z)-phycoerythrobilin as an incubation product was unexpected because *C. caldarium* does not contain phycoerythrin or other phycoerythrobilin-bearing phycobiliproteins. Further experiments indicated that *C. caldarium* extract contains an enzyme activity that converts (3Z)-phycoerythrobilin to (3Z)-phycocyanobilin.[6] The reverse reaction was not detected. The reaction does not require any substrate other than (3Z)-phycoerythrobilin. Because (3Z)-phycoerythrobilin and (3Z)-phycocyanobilin are isomeric, the enzyme can be considered to be an isomerase. In addition to being able to transform the (3Z) isomer, the protein fraction was also able to catalyze the conversion of (3E)-phycoerythrobilin to (3E)-phycocyanobilin. The enzyme activity was named phycobilin (15,16-methylene-to-18^1,18^2-ethyl) isomerase.

Cell extract proteins were partially fractionated by differential $(NH_4)_2SO_4$ precipitation, Blue-Sepharose affinity chromatography, and Sephadex G-75 gel filtration chromatography. The fraction containing proteins in the 30 000 to 40 000 molecular weight range were active in converting biliverdin to phycoerythrobilin but not to phycocyanobilin. The fraction containing proteins of greater than 60 000 molecular weight was inactive in biliverdin reduction, but catalyzed isomerization of phycoerythrobilin to phycocyanobilin.[6] These results suggest that phycoerythrobilin is an intermediate in the biosynthesis of phycocyanobilin from biliverdin.

4. Identification of 15,16-Dihydrobiliverdin IXα as an Intermediate

Partially fractionated *C. caldarium* protein extract, when incubated with biliverdin IXα and reductant, produced, in addition to phycocyanobilin and phycoerythrobilin, a third pigment which was identified by comparative absorption spectrometry and ^{1}H NMR as 15,16-dihydrobiliverdin IXα.[87] Further fractionation of the proteins by ferredoxin–Sepharose affinity chromatography yielded a fraction that formed 15,16-dihydrobiliverdin IXα as the only product of biliverdin IXα reduction. Purified 15,16-dihydrobiliverdin IXα, when incubated with another protein fraction, was converted to (3Z)-phycoerythrobilin and (3Z)-phycycyanobilin. This conversion, as well as the conversion of biliverdin IXα to 15,16-dihydrobiliverdin IXα, required reductant in addition to the bilin substrate. These results suggest that 15,16-dihydrobiliverdin IXα is a partially reduced intermediate in the biosynthesis of phycoerythrobilin from biliverdin IXα.

5. Enzymes Catalyzing the Reactions

Fractionation of the proteins catalyzing NADPH-dependent biliverdin reduction to phycobilins in *C. caldarium* extracts revealed that two of the components are the same ones that are needed for the heme oxygenase system: ferredoxin and ferredoxin–NADP$^+$ reductase.[89] As was shown earlier for heme oxygenase,[78] these components could be replaced with commercial counterparts from spinach or a red alga. Also, as with heme oxygenase, the requirements for NADPH and ferredoxin–NADP$^+$ reductase could be supplanted by a light-driven ferredoxin-reducing system derived from spinach thylakoids.[80] The results of Rhie and Beale[80] indicate that reduced ferredoxin is the only reductant needed for the reduction of biliverdin to phycobilins.

Protein fractionation results also indicate that the reduction of biliverdin to phycoerythrobilin proceeds by two two-electron steps, each of which is catalyzed by a different enzyme. One enzyme, when supplied with biliverdin IXα plus a source of reduced ferredoxin, produces only 15,16-dihydrobiliverdin IXα.[87] Further reduction of the bilin to phycoerythrobilin requires the presence of other proteins. The enzyme that catalyzes the first reduction step can be separated from the other reductase by affinity chromatography on ferredoxin–Sepharose.

As described above, the protein fractionation results suggest that the reduction steps are catalyzed by enzymes having apparent native molecular weights of approximately 30 000 to 40 000, and the isomerization of phycoerythrobilin to phycocyanobilin is catalyzed by an enzyme that has an apparent native molecular weight greater than 60 000.

In summary, biosynthesis of phycocyanobilin from heme in *C. caldarium* extracts is catalyzed by heme oxygenase to yield biliverdin IXα, followed by two two-electron reductions, which respectively yield 15,16-dihydrobiliverdin IXα and phycoerythrobilin, followed by isomerization of phycoerythrobilin to phycocyanobilin. Each step is catalyzed by a different enzyme. Both heme oxygenase and the bilin reduction steps are ferredoxin dependent. The extracts also contain an enzyme that isomerizes the 3-ethylidine group of phycoerythrobilin and phycocyanobilin from the *Z* configuration to the *E* configuration.

VI. Hypothetical Routes to Other Phycobilins

The number of known phycobilins continues to grow as a greater diversity of organisms is examined in detail. To date, of the eight known phycobilins (Figure 1), only three have been synthesized in vitro: 15,16-dihydrobiliverdin IXα, phycoerythrobilin, and phycocyanobilin. The existence of 15,16-dihydrobiliverdin as a phycobiliprotein chromophore[10] was actually anticipated by its discovery as an intermediate in the biosynthesis of phycoerythrobilin and phycocyanobilin.[87] Although experimental data on the biosynthesis of the other phycobilins are lacking, it is possible to arrange all of the known phycobilins in a general hypothetical biosynthetic pathway (Figure 8). The appeal of this pathway is the economy of reaction types: only five distinct reactions, acting on different substrates, can account for the formation of all known phycobilins plus the phytochrome chromophore. The hypothetical pathway provides direction for future research aimed at detecting the biosynthetic steps in vitro. Another consideration that should be borne in mind is that some phycobilins might be converted to others after ligation to apophycobiliproteins. Although there is no direct evidence to support this possibility, some indirect evidence exists which is consistent with it. For example, when a phycoerythrin-nonaccumulating mutant strain of *Calothrix* sp. PCC 7601 is grown under green light, which normally induces phycoerythrin synthesis in wild-type cells, a chromophore with a violin-like absorption spectrum appears on the α subunit of phycocyanin.[95] It was proposed that the violin-like chromophore is formed from protein-bound phycocyanobilin by an isomerase which, in wild-type cells, acts on phycoerythrin-bound phycocyanobilin to convert it to phycoerythrobilin. Although recent in vitro results (described above) indicate a different route for phycoerythrobilin formation, the concept is still valid and other phycobilin transformations may eventually be found to occur on ligated pigments.

VII. Phytochrome Chromophore Biosynthesis

The structure of the phytochrome chromophore very closely resembles those of the phycobilins.[96] Phytochrome is present in higher and lower plants and at least some algae, including several green algae and the red algae *Porphyra tenera*[97] and *Corallina elongata*.[98,99] Elich and Lagarias[100] obtained evidence that the phytochrome chromophore, like phycocyanobilin, can be synthesized in vivo from exogenously supplied biliverdin. These workers found that phytochrome levels are substantially reduced in oat seedlings germinated in the presence of gabaculine (3-amino-2,3-dihydrobenzoic acid), a potent, specific inhibitor of ALA formation via the five-carbon pathway. If either ALA or biliverdin was administered to the gabaculine-grown seedlings, there was a rapid increase in spectrophotometrically detected phytochrome. Label from ^{14}C-biliverdin was shown to be specifically incorporated into the phytochrome chromophore in oat leaves, thus establishing a clear biosynthetic link between this chromophore and the phycobilins.[101] More recently, phytochrome-deficient mutant strains of *Arabidopsis thaliana* that are defective in chromophore biosynthesis were demonstrated to be able to synthesize functional, phototransformable phytochrome when biliverdin IXα was administered.[102] Thus, the experimental evidence indicates that phytochromobilin can be considered to be one of the end products of the general phycobilin biosynthetic pathway (Figure 8). It can be hypothesized that, in organisms that synthesize phytochromobilin, the 2,3-reductase enzyme is able to accept biliverdin IXα as the substrate, whereas in species that synthesize phycobilins and not phytochromobilin, this enzyme instead accepts 15,16-dihydrobiliverdin as the substrate (Figure 8).

VIII. Ligation of Bilins to Apoproteins

All phycobilins exist, in their functional state, as covalently attached chromophores of the phycobiliproteins. Until very recently, there was no direct information available concerning the mechanism by which phycobilin chromophores are ligated to the cysteine sulfhydryl group(s) of the apoproteins. Relatively little is known about the closely analogous

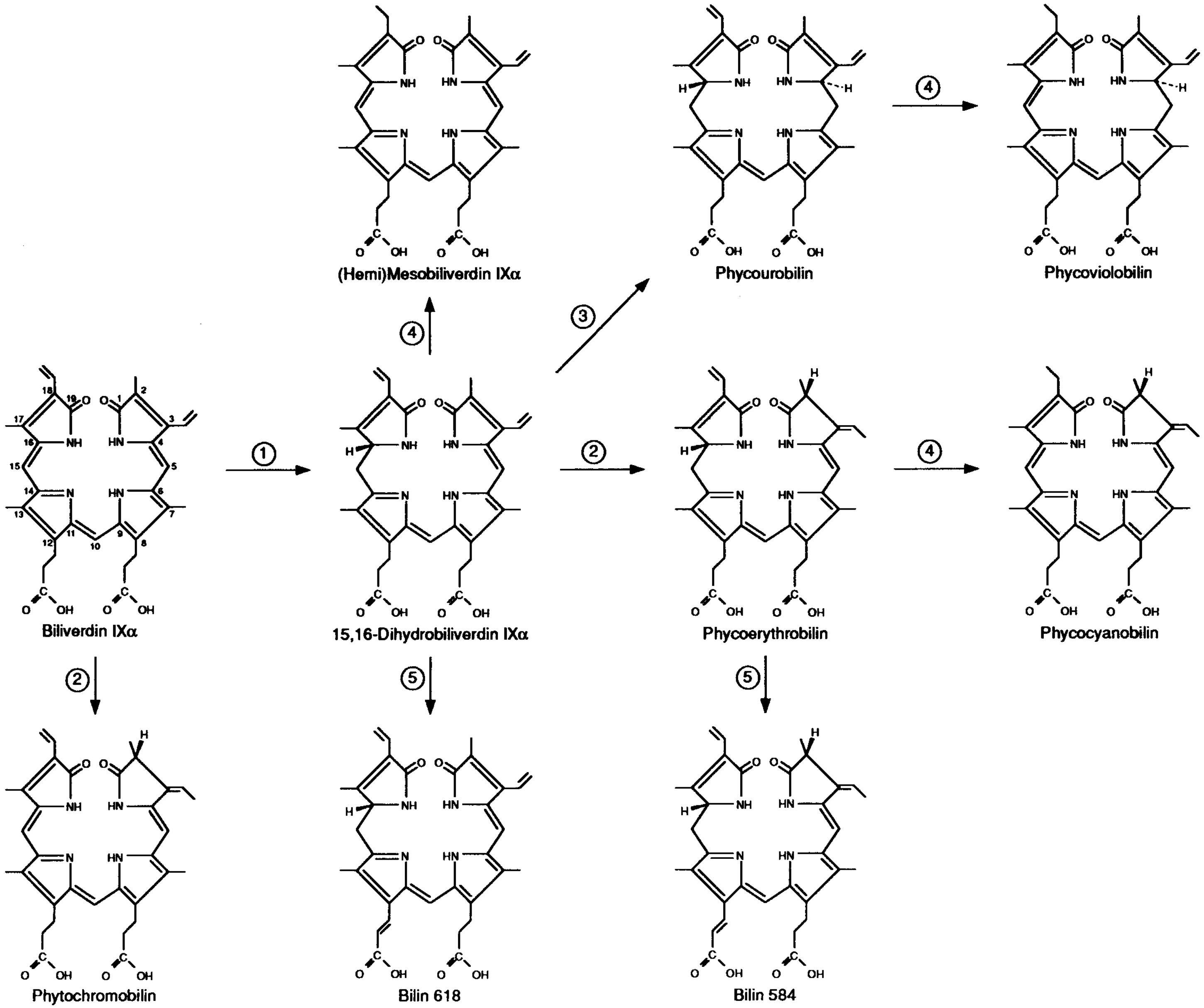


Figure 8. Hypothetical general pathway for phycobilin and phytochromobilin biosynthesis. Phytochromobilin and all of the known phycobiliprotein chromophores can be derived from biliverdin IXα by only five reactions, acting on various substrates. The five reactions are (1) biliverdin 15,16-reductase, (2) bilin 2,3-reductase, (3) phycobilin 4,5-reductase, (4) phycobilin (15,16-methylene-to-18^2,18^3-ethyl) isomerase, and (5) phycobilin 12^1, 12^2-dehydrogenase. The ethylidine groups, when present, are shown in the *Z* configuration. One or more (*Z*-to-*E*) ethylidine isomerases catalyze the conversions to the (3*E*)-ethylidine isomers, which may be the immediate precursors of the protein-bound chromophores (see text). The conventional bilin numbering system is shown for biliverdin IXα. The unbound form of the mesobiliverdin chromophore is actually a hemimesobiliverdin because it contains one vinyl and one ethyl substituent.

process of heme ligation to apoprotein during cytochrome *c* synthesis. The latter reaction is catalyzed by a mitochondrial particulate enzyme called cytochrome *c* synthase.[103] In vitro activity of the enzyme derived from yeast was stimulated by pyridine nucleotides and by a soluble, heat-stable factor. More recently, a detergent-solubilized, partially-purified heat-labile enzyme has been obtained from yeast mitochondria, which forms a heme–apocytochrome adduct having spectral characteristics identical to authentic cytochrome *c*, when incubated with the two substrates plus NADPH.[104] The NADPH functions to keep the heme iron in the ferrous oxidation state, which is apparently required for ligation.[105] The enzyme has been localized within the inner mitochondrial membrane.[106] Patterns of cross-species acceptance of apoprotein substrate by cytochrome *c* synthases from yeast and beef heart suggest that the enzymes recognize specific peptide sequences on the apoproteins and position the heme to generate the correct linkages.[107] A synthetic peptide corresponding to the first 25 amino acids of horse cytochrome *c* effectively incorporated heme into a reactive center when incubated with a yeast mitochondrial fraction containing the thioether bond-forming enzyme activity.[108] Genes coding for cytochrome *c* synthase have been cloned by complementation of cytochrome *c*-deficient yeast[109] and *Neurospora*[11] mutants, and the genomic sequences have been determined.

A. Phycocyanin

Attempts were made to reconstitute native phycocyanin in vitro by combining apoprotein and free bilins.[111–113] The source of the apoprotein was the cloned genes encoding the α and β subunits of phycocyanin from *Synechococcus* sp. PCC 7002 (*Agmenellum quadruplicatum* PR-6) that were expressed in *Escherichia coli*.[114] Although covalent binding of phycocyanobilin to cysteine residues occurred, the binding appeared to be nonspecific and did not yield native holoprotein. Moreover, the bilin had been altered during the binding reaction, and the bound chromophore lacked a reduced pyrrole ring, i.e., it had the structure of mesobiliverdin IXα.

Recent genetic and biochemical results support a specific role for certain proteins in phycobilin ligation.[115,116] At the *cpc* gene locus of *Synechococcus*, there is, in addition to genes that code for phycocyanin apoprotein subunits, two open reading frames designated *cpcE* and *cpcF*. Insertional mutagenesis of *cpcE* or *cpcF* caused the appearance of yellow-green cells that failed to accumulate normal amounts of phycocyanin. The cells contained normal phycocyanin β subunits, but the α subunits lacked chromophores. These results were interpreted to indicate that the ligation of the chromophore to the α subunit is catalyzed by the *cpcE* and *cpcF* gene products. Recombinant CpcE and CpcF proteins were overproduced in *E. coli*. Together, these proteins catalyzed in vitro ligation of phycocyanobilin to the α subunit at the correct cysteine residue (Cys-84).[117] Spectroscopy indicated that, unlike the case when CpcE and CpcF were absent, the CpcE/CpcF-assisted ligation product had bound phycocyanobilin rather than mesobiliverdin. In addition to catalyzing ligation of free phycocyanobilin, CpcE/CpcF catalyzed exchange of bilin from the α-subunit of phycocyanin to resin-bound α-apophycocyanin.

Phycocyanobilin binds tightly to CpcE/CpcF. The kinetics of the CpcE/CpcF-enhanced ligation of phycocyanobilin to α-apophycocyanin showed a rapid initial bilin ligation phase followed by a slower linear phase. The kinetics were interpreted to indicate that CpcE/CpcF catalyzed the rapid reaction of a minor conformer or configurational isomer of phycocyanobilin with the apoprotein. The subsequent slow phase represents further ligation of the appropriate form of phycocyanobilin as it is produced by slow isomerization of the bulk of the remaining phycocyanobilin. A possible structure of the faster-reacting bilin is (3*Z*)-phycocyanobilin. This species exists as a minor component as a methanolysis product of phycocyanin[5] and is the major product of in vitro phycocyanobilin formation from biliverdin in *C. caldarium* cell extracts.[5,89] *C. caldarium* cell extracts contain an enzyme that catalyzes GSH-dependent cis–trans isomerization of the 3-ethylidine group of phycocyanobilin.[89] It will be of interest to determine if the ethylidine cis–trans isomerase is encoded by the *cpcE* and/or *cpcF* genes.

Because the CpcE/CpcF proteins appear to affect phycocyanobilin attachment only to the α-phycocyanin subunit, there are presumably other, so far undiscovered proteins that are required for phycobilin ligation to β-apophycocyanin and other apophycobiliproteins. It is unclear how the different chromophores and apoprotein ligation sites are recognized by the components responsible for attaching the various different chromophores at specific sites. The ligation process is further complicated by the recent discovery that although ligation results in the generation of an α-thioethyl group with an *R* configuration at most sites, at some sites the thioethyl group has the *S* configuration.[16] Furthermore, recently it has become apparent that the thioether links do not invariably involve the α-carbon of the bilin substituent: certain doubly-linked 15,16-dihydrobiliverdin chromophores of cryptomonad phycobiliproteins have been shown to be attached to the apoproteins at the β-carbon of the bilin 3-position substituent and at the α-carbon of the bilin 18-position substituent, i.e., the linkages are 3″ and 18′.[118]

B. Phytochrome

In contrast to the complex ligation requirements for phycobiliproteins, reconstitution of phytochrome appears to be simple and spontaneous. Elich et al.[101] were able to measure in vivo incorporation of exogenous biliverdin and phycocyanobilin into phytochrome in etiolated oat leaves that had been prevented from synthesizing endogenous bilins by administration of the inhibitor of ALA formation, gabaculine. After incubation of the gabaculine-treated leaves with exogenous bilins, the phytochrome was isolated and found to undergo photoreversible spectral changes. In the case of phycocyanobilin administration, the isolated phytochrome had its absorption maxima shifted as a result of incorporation of a bilin other than the natural one, phytochromobilin. Later, in vitro reconstitution of apophytochrome was attempted with free phycocyanobilin. The source of apophytochrome was oat seedlings grown in the presence of 4-amino-5-hexynoic acid, a powerful inhibitor of ALA synthesis,[119] or synthetic apophytochrome derived by in vitro transcription and translation of cloned oat phytochrome cDNA.[120] Similar reconstitution experiments were also reported for phycocyanobilin with recombinant pea phytochrome expressed in yeast.[121] The covalently linked products underwent photoreversible spectral changes and had an absorption spectrum indicative of apophytochrome-linked phycocyanobilin. Biliverdin did not react with apophytochrome in vitro, suggesting that a bilin possessing a reduced pyrrole ring and/or an ethylidine group is essential for the reaction to occur. Also, the fact that phycocyanobilin does not condense with simple thiol-containing compounds,[111-113] whereas the reaction with apophytochrome appears to be spontaneous, was interpreted to indicate that in the condensation with apophytochrome, the apoprotein serves as a ligating enzyme.[119]

More recently, recombinant oat apophytochrome was reconstituted with phytochromobilin to yield a photoreversible product with spectral properties identical to that of native oat phytochrome. In some experiments, phytochromobilin was synthesized in situ by cucumber cotyledon etioplasts that were incubated with biliverdin IXα.[122,123] The product was presumed to be phytochromobilin but it was not isolated or characterized. In later experiments, the source of authentic, chemically and spectrally characterized phytochromobilin for reconstitution was the unicellular red alga, *Porphyridium cruentum*, whose phycoerythrin chromophores can apparently be oxidized to phytochromobilin while the reduced pyrrole ring is protected from oxidation by the attachment to the protein.[84] Subsequent cleavage of the protein–chromophore thioether link by methanolysis and purification by solvent partitioning, DEAE–Sepharose ion exchange chromatography, and high-pressure liquid chromatography yielded purified (2*R*,3*E*)-phytochromobilin, whose structure was verified by comparative absorption (Table I), circular dichroism, and ^{1}H NMR spectroscopy.[84] It was also shown that phycoerythrobilin will spontaneously condense with apophytochrome, but in this case, the product is not photoreversible.[124] The relative rates of condensation of the three chromophores with apophytochrome was phytochromobilin > phycocyanobilin ≫ phycoerythrobilin.[124]

The relative ease of in vitro phytochrome reconstitution compared to phycocyanin reconstitution may indicate that the most abundant free bilin species, e.g., the (*E*)-ethylidine isomer, is the immediate precursor to the bound form of the phytochrome chromophore. Alternatively, apophytochrome itself may catalyze any bilin alteration that is required for ligation. Apophytochrome, a 124-kDa peptide, is much larger than apophycobiliproteins, which are in the 15-kDa range, and it may carry the bilin-modifying function that is catalyzed by CpcE/CpcF in α-phycocyanin ligation.

In summary, ligation of bilin with apophytochrome to form native holoprotein is spontaneous, involves no enzyme function other than what may occur on apophytochrome, and requires a bilin having a reduced pyrrole ring and an ethylidine group. Assembly of native phycocyanin is more complicated and involves other proteins which may function as ligation enzymes and/or bilin modifiers. Different subsidiary proteins appear to be required for each ligation position.

IX. Regulation of Phycobilin and Phycobiliprotein Formation

Phycobiliprotein-containing organisms are able to modulate their cellular content of these pigments in response to the nutritional environment and the quantity and spectral composition of the light environment.[125] In some cases, the relative composition of the pigment repertory is responsive to the spectral quality of the light, a phenomenon known as complementary chromatic adaptation.[126,127] At present, neither the extent to which phycobilin biosynthesis is regulated by factors that affect phycobiliprotein formation nor the effects of phycobilins on phycobiliprotein formation are known. The following summary is intended to provide a framework for guiding future study of these interrelated questions.

Phycobiliproteins comprise a significant proportion of total cellular proteins of cyanobacteria when the cells are grown in medium containing nonlimiting amounts of nitrogen. Cells stop synthesizing phycobilins when nitrogen becomes limiting. In *Synechococcus*, it has been shown that the *cpc* promoter, which regulates expression of the phycocyanin apoprotein genes, is responsive to the cellular nitrogen status. mRNA encoding the α and β subunits of phycocyanin is undetectable in nitrogen-limited cells. By using plasmids containing the *lacZ* gene under regulation by the *cpc* promoter, it was shown that *lacZ* expression is rapidly diminished after the cells are shifted to low nitrogen medium, and expression is regained shortly after resupplying nitrogen.[128] Nitrogen starvation not only prevents phycobiliprotein synthesis, but it also induces the synthesis of a protease that degrades phycobiliproteins in *Anabaena cylindrica*.[129] Because the proteolytic activity against phycocyanin was not diminished in the presence of much higher concentrations of bovine serum albumin, the protease was considered to be specific for phycocyanin and was named phycocyaninase. An apparently similar protease is induced in *Anabaena* cells by conditions that induce heterocyst differentiation.[130] Induction of the protease was blocked by chloramphenicol. Although absolute specificity for phycocyanin was not established, the protease appeared to be more active against phycocyanin than other soluble proteins.

In a wide range of photosynthetic species, there is observed an inverse relation between the incident light intensity and the concentration of light-harvesting pigments.[131] The "shade" phenotype is characterized by a high content of light-harvesting pigments relative to reaction centers and other components of the photosynthetic apparatus. In cyanobacteria and red algae, the cellular content of phycobiliproteins is inversely related to the incident light intensity. It was shown that in *Synechococcus* sp. PCC 6301, the shade phenotype can be induced even in high light by administration of sublethal concentrations of the inhibitor of photosynthetic electron transport, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.[132] It thus appears that the adaptation to light intensity in cyanobacteria is based on cellular monitoring of the rate of photosynthesis, which is perhaps signaled by the intracellular concentration of some photosynthetic product. A similar mechanism was suggested earlier for light intensity adaptation in the unicellular green alga *Chlorella vulgaris*, on the basis of the responses to administered sugars and inhibitors of photosynthetic electron transport.[133]

In addition to regulation by light intensity, wavelength-sensitive light regulation has also been demonstrated in the unicellular red alga *C. caldarium*. Wild-type cells of this species require light for chlorophyll and phycocyanin synthesis. Nichols and Bogorad[134] examined the action spectrum for phycobilin formation in a nonphotosynthetic, chlorophyll-less mutant of *C. caldarium*. Light of 420-nm wavelength was most effective in promoting phycocyanin formation. A second peak in the action spectrum occurred at about 580 nm. The authors proposed that the photoreceptor may be a heme compound. Dark phycocyanin synthesis could be induced by unidentified factors present in some culture medium components. Another mutant strain of *C. caldarium* accumulates chlorophyll *a* but not phycocyanin. The action spectrum for chlorophyll synthesis corresponds to the absorption spectrum of protochlorophyllide.[135] Wild-type cells, which contain both chlorophyll and phycocyanin, have a single action spectrum for synthesis of both pigments, and this resembles the sum of the action spectra exhibited by the two mutant strains.

The cellular levels of the α and β subunits of phycocyanin in *C. caldarium* appear to be regulated at the transcriptional level by heme.[136,137] Wild-type cells do not accumulate phycobiliproteins in the dark, and the plastid mRNAs coding for the apoproteins are also absent in the dark.[138–140] However, if the cells are administered the phycobilin precursors ALA, protoporphyrin, or heme, in the dark, they accumulate phycocyanin and allophycocyanin apoproteins and mRNAs. Other genes (photogenes) which are normally expressed only in the light, were not induced in the dark by phycobilin precursors. Biliverdin, which is also a phycobilin precursor, did not stimulate phycobiliprotein apoprotein or mRNA accumulation in the dark. The authors concluded that heme is a specific regulatory factor involved in the transcriptional regulation of phycobiliprotein gene expression.

When cloned cyanobacterial apophycocyanin genes were reintroduced into *Synechococcus* at higher multiplicity, overexpression of the genes resulted. The cells accumulated correspondingly greater than normal amounts of chromophore-containing phycocyanin.[141] Apparently, the cells were able to synthesize sufficient phycobilins to accommodate the increased demand for pigment. This result suggests that bilin formation does not limit the rate of phycocyanin accumulation, but that, instead, the bilin biosynthetic rate may respond to the availability of apoprotein.

In cyanobacteria that contain both phycocyanin and phycoerythrin, at least three mechanisms are known to operate in bringing about changes in phycobilin pigment composition during complementary chromatic adaptation. Some species vary the ratio of phycoerythrin to phycocyanin.[142] Other species contain two classes of phycocyanin, one of which is expressed constitutively and is formed in red or green light, while the other is inducible and is formed only in red light.[143] Still other organisms have been reported to incorporate different bilins into apoprotein subunits under different growth conditions.[144]

Since phycobiliproteins differ from each other in the protein moieties as well as in their phycobilin complement, differential synthesis or breakdown of proteins must be a requirement for complementary chromatic adaptation. Gendel et al.[145] found that the induction of phycoerythrin synthesis by green light in the cyanobacterial species *Calothrix* sp. PCC 7601 (*Fremyella diplosiphon*) is blocked by rifamycin, indicating a requirement for gene transcription in the response. Transfer from green light to darkness slowed, but did not stop, phycoerythrin synthesis, indicating that differential phycobiliprotein synthesis is induced by green light, but that continuous irradiation is not required for the response to occur. Cells that were transferred from green to red light stopped synthesizing phycoerythrin within 45 min. The same time course for cessation of phycoerythrin synthesis was exhibited when rifamycin was administered to cells growing in green light, suggesting that the chromatic adaptation response is mediated at the transcriptional level.[145] More recently, it was demonstrated that green light induces transcription of the phycoerythrin operon in *Calothrix*.[56]

A search for pigments that might mediate the response to variations in spectral light distribution has resulted in the detection of several photochromic species, both in vivo and in vitro.[146] Isolated photochromic pigments appear to be similar or identical to some phycobiliprotein subunits. They undergo photoreversible transformations, with peak positions that are close to the peaks of the action spectra for the chromatic adaptation responses, i.e., 520–550 nm for the green light effects and 640–660 nm for the red light effects.[147] The photochromic species were named phycochromes by Björn and Björn.[148] Although some authors have considered phycochromes to be artifacts that arise from partial degradation of allophycocyanin during extraction of the cells,[149,150] recent studies have clarified the relationship of photochromic pigments to phycobiliproteins (see below). Because of their close structural and functional resemblance to phytochrome, the phycochromes are attractive candidates for evolutionary predecessors.

The photochromic pigment in several cyanobacterial species has been identified as the α subunit of phycoerythrocyanin.[151–153] This subunit bears a single phycoviolobilin chromophore at Cys-84. Free α subunit, but not the β subunit or the native phycoerythrocyanin holoprotein, undergoes reversible photochromic changes that are indicative of phycochrome *b*, with difference maxima at 503 and 570 nm.[153] Not only does the state of aggregation of the subunit affect the photochromism, but the state of the chromophore conversely affects the state of aggregation. This effect was proposed as a possible primary signal whereby the photochromic release of polypeptides constitutes an intracellular signal.[152] Recently, peptides bearing 15,16-dihydrobiliverdin IXα chromophores were also found to be photochromic.[10]

Beguin et al.[95] described a mutant strain of *Calothix* that does not accumulate phycoerythrin under any light regime. In red light, the cells are phenotypically indistinguishable from wild-type. In green light, some of the phycocyanin subunits contain a bilin of unknown structure, which has a distinctive violin-like visible absorption spectrum. These interesting observations have been interpreted as supporting the hypothesis that bilins other than phycocyanobilin are derived from protein-bound phycocyanobilin, either by enzymatic isomerization or nonenzymatically as a result of protein folding.[95] However, a clearer understanding of these results may have to await determination of the structure of the novel bilin and the nature of the defect in the mutant cells.

In cells that are accumulating chlorophyll, hemes, and phycobilins, the first biosynthetic step unique to the phycobilin branch would be the heme oxygenase reaction. This reaction is therefore a logical candidate for a physiological control point, regulating the cellular level of phycobilins. However, it remains to be demonstrated whether heme oxygenase or any other enzyme specific to phycobilin biosynthesis is regulated by light or nutritional status of the cells. At present, the only study at the enzyme level to reveal modulation of the activity of a step of phycobilin synthesis is that of Brown et al.,[154] which reported a large increase in the activity of ferrochelatase paralleling the rise in phycocyanin accumulation when dark-grown wild-type *C. caldarium* cells were transferred from the dark to the light. It remains to be determined whether the increase in ferrochelatase level in turn regulates the rate of phycocyanobilin synthesis.

X. Outlook

Recent progress in understanding phycobilin biosynthesis includes identification of biosynthetic precursors and intermediates including heme and biliverdin, demonstration of a biosynthetic relationship among several phycobilins including 15,16-dihydrobiliverdin, phycoerythrobilin, and phycocyanobilin, and successful in vitro reconstitution of phytochrome and the α subunit of phycocyanin. Some progress has been made toward characterizing the enzymes catalyzing the heme oxygenase reaction, biliverdin reduction, and ethylidine isomerization including the demonstration that ferredoxin is the immediate source of the electrons for heme oxygenation and biliverdin reduction. It can be expected that in the near future, the biosynthesis of other phycobilins will be elucidated, the enzymes responsible for phycobilin synthesis will be further characterized, the requirements for bilin-apoprotein ligation will be determined for other proteins, and the role, if any, of the biosynthesis of phycobilins in regulating the cellular content and composition of phycobiliproteins will be demonstrated.

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XII. References

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