Cyanobacterial Phycobilins: Production, Purification, and Regulation

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Abstract

The cyanobacteria are a diverse group of prokaryotic organisms that carry out oxygenic photosynthesis and are thought to be responsible for the oxygenation of our atmosphere. Like red algae and cryptomonads, cyanobacteria also contain phycobiliproteins (PBPs) which serve as major accessory pigments during photosynthesis. PBPs are large water-soluble supramolecular protein aggregates involved in light harvesting and can be divided broadly into three classes, viz., phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) based on their spectral properties. PBPs have been extracted and purified from *Spirulina* spp., *Synechococcus* sp., *Oscillatoria* sp., etc., and produced commercially from *Spirulina platensis, Anabaena* sp., and *Galdieria sulphuraria*. Since cyanobacteria exhibit wide variations in nutrient availability, light intensity, light quality (wavelength), temperature, water activity, etc., these variations also result in altered metabolic activity of these organisms as a result of differential expression of different genes. The expression of phycobiliprotein coding genes is also accordingly modulated to adapt to a particular condition. Many workers have reported changes in phycobilisome structure and expression of *cpc* genes in response to light quality, light quantity, and nutrients like nitrogen, sulfur, etc. The composition and function of phycobiliproteins in cyanobacteria have also been reported to change under stress conditions. In the present paper, we have reviewed the production, purification, and regulation of cyanobacterial phycobilins including their importance in the commercial sector, as they have several applications as natural dyes in food and cosmetic industry, immunological assays, health-promoting properties, and broad range of other pharmaceutical applications.

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The cyanobacteria constitute a kingdom of the domain bacteria whose members manifest a vast diversity of habitats and metabolisms. Species differ with respect to possession of panoply of specializations, a sampling of which includes diazotrophy, filamentous growth form, production of buoyancy organelles, and differentiation of specialized cell types. However, the group can be defined by the unifying property of chlorophyll *a*-based oxygenic photosynthesis which is considered as the source of the earth's oxidizing atmosphere and extends to plants, whose chloroplasts trace their ancestry to this kingdom (Olsen et al.1994; Woese 1987). Unlike the higher plants, cyanobacteria possess phycobiliproteins as major light-harvesting pigments which are also found in red algae and glaucophytes. Due to the presence of blue-colored biliprotein phycocyanin, many of the cyanobacteria appear bluish-green in color and they have been named as blue-green algae despite their prokaryotic nature. Phycobiliproteins play important role in cyanobacterial metabolism as well as help them to adapt in a diverse environmental condition especially conditions with variable light quality and quantity. These proteins due to their multifunctional role in cyanobacterial survival and versatile chemical properties have been very interesting subject of research particularly for the algologists and biochemists. Because of their attractive color and stable fluorescence property, these proteins have been exploited in food as well as pharmaceutical industries, and a number multinational companies all over the world are engaged in commercial production of phycobiliproteins and related products.

Some of the major aspects of phycobiliproteins have been reviewed earlier by Bogorad (1975), Zilinskas and Greenwald (1986), Grossman et al. (1988), and Glazer (1994); however, these reviews did not discuss many of the important and interesting aspects of phycobiliproteins like their biosynthesis, modulation of their production by environmental conditions, their purification, and most importantly their application.

4.1 Phycobilisomes: Structure, Components, and Biosynthesis

Phycobilisomes (PBsomes) are protein assemblies that function in light harvesting and energy transfer, usually to PS II. When the energy absorbed by the PBsomes reaches the reaction centers of PS II, excitation energy is transduced to chemical energy. These biliproteins also known as phycobiliproteins absorb radiations in regions of the visible spectrum where *Chl*a has low absorptivity. Depending on the bilin energy, phycobiliproteins are categorized into three types: phycoerythrins with high energy (λ_{max} ~565 nm), phycocyanins with intermediate energy (λ_{max} ~620 nm), and allophycocyanins with low energy (λ_{max} ~650 nm). The energy flows from high-energy to low-energy pigments and phycobiliproteins are organized accordingly. Six rods in hemidiscoidal PBsomes have three cylinders in their core which is situated near the thylakoid membrane and PS II, where chlorophyll *a* is located. The rods have the phycoerythrin farthest from the core. The core has the allophycocyanins, of which there are two functional types: allophycocyanin with absorbance maxima of 650 nm and two other lower-energy allophycocyanins, the L_{CM} polypeptide and the α^B polypeptide, involved in energy transfer to chlorophyll (Gantt 1975; MacColl and Guard-Friar 1987; Bryant et al. 1979; Sidler 1994; Bald et al. 1996). The rods are built with stacks of disks, and the disk adjacent to the core is invariably phycocyanin.

Phycobiliproteins are commonly found to consist of hetero-monomers which are composed of two different subunits, α and β (Gantt 1980; Glazer 1986; Zilinskas and Greenwald 1986; Rowan 1989). These subunits are present in equimolar ratio (α:β::1:1) and differ in molecular mass, amino acid composition, and chromophore content. Fundamental configuration of the phycobiliprotein assembly is a stable trimer $(\alpha\beta)_3$ forming a toroidal-shaped aggregate (Sidler 1994; MacColl 1998; Tandeau de Marsac 2003; Adir 2005). The aggregate is 3–3.5 nm thick with a radius of about 5.5 nm and a central hole of 3 nm in diameter. Some biliproteins exist in hexamers, such as PE by face-to-face aggregation of two trimeric disks in the central cavity (Sidler 1994; MacColl 1998; Tandeau de Marsac 2003; Adir 2005; Glazer 1989; Ducret et al. 1996, 1998). Phycobiliproteins are brilliantly colored due to covalently bound open-chain tetrapyrrole chromophores named phycobilins. They are either blue-colored phycocyanobilin (PCB), red-colored phycoerythrobilin (PEB), the yellow-colored phycourobilin (PUB), or the purple-colored phycoviolobilin (PVB), also named cryptoviolin. These chromophores are attached to the polypeptide chain at conserved sites either by one or two cysteinyl thioester linkage (Glazer 1985).

4.1.1 Phycoerythrin (PE)

PE is the most abundant PBP in many red algae and in some cyanobacteria. These are characterized by strong absorption in the green region (480–570 nm) and by intensive fluorescence emissions at about 575–580 nm. Based on their absorption spectra, these red-colored proteins fall into three distinct species: (1) B-phycoerythrin (B-PE) (λ_{max} ~540–560 nm, shoulder ~495 nm), (2) R-phycoerythrin (R-PE) (λ_{max} ~565, 545 and 495 nm), and (3) C-phycoerythrin (C-PE) (λ_{max} ~563, 543 and ~492 nm). The prefixes B-, R-, and C- were used historically for indicating the type of organisms from which the pigment proteins were originally extracted, for example, Bangiales, Floridian Rhodophyceae, and Cyanophyceae. C-PEs are most abundant phycoerythrins in cyanobacteria. They are the products by some cyanobacteria that acclimate to their environment variations in light quality and intensity, especially green-to-red light ratios, through a process traditionally called complementary chromatic adaptation (Ohki et al. 1985; Toledo et al. 1999; Grossman et al. 2001; Palenik 2001; Misckiewicz et al. 2002; Grossman 2003; Everroad et al. 2006). These cyanobacteria make more of the light-harvesting protein phycocyanin in red light and more of the protein phycoerythrin in green light (Rowan 1989; Sidler 1994; MacColl 1998; Toledo et al. 1999; Grossman et al. 2001; Palenik 2001; Misckiewicz et al. 2002; Grossman 2003; Everroad et al. 2006). There are two subtypes of C-PE, viz., C-PE-I or PE (I) and C-PE-II or PE (II). The PEs from freshwater and soil cyanobacteria typically contain only PEBs and exhibit absorption spectra with maxima at about 565 nm and fluorescence emission spectra with peaks at about 575 nm. These are the spectral properties characteristic of C-PE-I (or PE (I)), also known as C-PE. C-PE-I usually carries five PEBs per monomer (αβ) (Rowan 1989; Sidler 1994; MacColl 1998; Ong and Glazer 1991). The five PEBs are covalently attached to the cysteine residues, in the way very similar to B-PE, at α -84, α -140/143, β -84/82, β -50/β -61, and β -155/159, respectively. The α and β subunits vary with their origins in molecular mass from 15 to 22 kDa. C-PE-I is a disk-shaped hexamer, which is constructed by two trimeric $(\alpha\beta)_3$ through face-to-face aggregation, commonly exists in form $(\alpha\beta)_6L_R$ where a rod-linker polypeptide (L_R) is bound to the central hole of the two stacking trimers. Unlike the γ subunit from B-PE and R-PE, however, the L_R of C-PE-I generally carries no phycobilins (Sidler 1994; MacColl 1998; Ong and Glazer 1991).

4.1.2 Phycocyanin

Phycocyanins are one of the most widespread phycobiliproteins observed in almost all phycobiliprotein-containing organisms, including cyanobacteria, red algae, glaucophytes, and

some cryptophytes. They are most abundantly found in most cyanobacterial species that grow in natural environment. The phycocyanin from the PBsome-containing algae is subdivided into three types: (1) C-phycocyanin (C-PC, λ_{max} ~615–620 nm) exclusively existing in cyanobacteria, (2) phycoerythrocyanin (PEC, λ_{max} ~575 nm) inducible only in some cyanobacteria, and (3) R-phycocyanin (R-PC, λ_{max} ~615 nm) mainly in red algae (Gantt 1980; Glazer 1986; Zilinskas and Greenwald 1986; Rowan 1989; Reuter and Muller 1993; Sidler 1994; MacColl 1998; Tandeau de Marsac 2003; Adir 2005). These blue- or blue-purple-colored phycobiliproteins have strong light absorption ability mainly in range from 580 to 630 nm and emit intensive red fluorescence at 635–645 nm. Phycocyanins exist commonly as trimer $(\alpha\beta)_3$ from aggregation of three (αβ) monomers. They have a thickness of 3 nm, a diameter of 11 nm, and a central hole in diameter of 3.5 nm. In PBsomes, phycocyanins commonly occur in disk-shaped hexamers, and a rod-linker polypeptide (L_R) or a rod-core-linker polypeptide is attached to the center cavity of hexamers to form $(\alpha\beta)_6L_R$ or $(\alpha\beta)_6L_{RC}$ complexes (Glazer 1989; Reuter and Nickel-Reuter 1993; Sidler 1994; Pizarro and Sauer 2001). The interaction of phycocyanins with the linker polypeptides may typically cause a large red shift of about 17 nm in their absorption and fluorescence emission maxima (Sidler 1994; Pizarro and Sauer 2001). C-phycocyanin (C-PC) is a blue-colored, deeply red-fluorescent phycobiliprotein and a predominant form among phycocyanins which is also one of the most studied PBPs. The C-PC subunits have globin-like folding, and according to the myoglobin nomenclature of helices, the eight helices of a C-PC subunit are denoted as X, Y, A, B, E, F, G, and H (Schirmer et al. 1985). During evolution, the oxygen-binding proteins of higher organisms (oxygen consumption in higher organisms) and PCs of oxygen-producing prokaryotes might have diverged from a common protein (Pastore and Lesk 1990). In a C-PC protein, three phycocyanobilin chromophores are carried by each (αβ) monomer. The three PCBs have been determined to bind covalently to certain conserved cysteine residues at Cys α-84, β-84, and β-155. Thus one C-PC in trimer carries three α-PCBs and six β-PCBs, and in hexamer it contains six α-PCBs and 12 β-PCBs. Investigation of energy transfer between chromophores in C-PC trimers has demonstrated that α-84 PCB and β-155 PCB function as the two sensitizing chromophores located at the periphery of C-PC trimers, whereas β-84 PCB works as the fluorescing pigment located near the central cavity of C-PC trimers and hexamers. In other words, the α-84 PCB and β-155 PCB in C-PCs function as the excitation energy transfer donors and the β-84 PCB as the terminal acceptors (Sidler 1994; Debreczeny et al. 1995a, b; MacColl 1998; Eisele et al. 2000; Kikuchi et al. 2000).

Amino acid sequences of the apoproteins and/or chromophore composition have varied with adaptation process to light and nutrient conditions. One, or in some cases two, of the peripheral sensitizing chromophores (α-84 PCB and/or β-155 PCB) have been replaced by PVB, PEB, or PUB chromophores in each of the variants in order to adapt to conditions more enriched in blue and green wavelengths of light. The fluorescing β-84 PCB was, however, conserved in all variants.

4.1.3 Allophycocyanin

Allophycocyanins (APCs) are a type of core-constructing phycobiliproteins. These are a less-contained phycobiliprotein species with respect to PCs and PEs, but they exist in all PBsome-containing organisms, cyanobacteria, glaucophytes, and red algae, which grow in natural environment. Compared with red algae, cyanobacteria may generally contain higher amounts of APCs with respect to total phycobiliprotein content of the organisms. A PBsome core is commonly composed of three kinds of allophycocyanins that are known as allophycocyanin (APC), allophycocyanin-B (APC-B), and allophycocyanin core-membrane linker (L_{CM}) complex (APC-L_{CM}) (Gantt 1980; Glazer 1986, 1989; Zilinskas and Greenwald 1986; Rowan 1989; Sidler 1994; MacColl 1998). Among these three, APC constitutes the largest number of core biliprotein components, whereas APC-B and APC-L_{CM} have equal copies in the core. In the PBsome core construction, APC participates in the assembly of all the

core cylinders, but APC-B and APC-L_{CM} take part merely in the two basal cylinders. Among the three types of allophycocyanins, the APC is the best described one. It exists in trimer of hetero-monomers (αβ) in PBsomes and in solution when extracted out of organisms. The APC trimers $(\alpha\beta)_3$, like PCs, show a threefold symmetry of the disk-shaped structure in crystal (Sidler 1994; Brejc et al. 1995; MacColl 1998; Liu et al. 1999). The APC $(\alpha\beta)_3$ in the disk-shaped conformation shows about 11 nm in diameter and 3 nm in thickness, and it has a central cavity of about 3.5 nm in diameter (Brejc et al. 1995; Liu et al. 1999). The α subunit consists of 160 amino acid residues, and the β subunit is composed of 161 amino acids (Troxler et al. 1980; Brejc et al. 1995; Liu et al. 1999); they exhibit apparent molecular masses in range from 17 to 20 kDa (Zilinskas 1982; Zilinskas and Greenwald 1986; Sidler 1994; MacColl 1998). One (αβ) monomer of the trimer contains two PCBs where one is attached at α-84 and the other at β-84. In the APC trimer, the β-84 PCB is situated near the central cavity, whereas the α-84 PCB is near the peripheral. The PCBs in the monomers were found to have an absorption maximum at 614 nm, but in the trimers they exhibited a sharp maximum at 650 nm and a prominent shoulder from 610 to 620 nm. It has been believed that one of the PCBs in the monomer, more probably β-84 PCB, is changed by interaction with the apoproteins in the trimer construction and that the PCB then creates the 650 nm absorption maximum owing to its unique conformation or a different environment (Sidler 1994; MacColl 1998, 2004). The other PCB, more possibly α-84 PCB that retains its original conformation similar to what it has in the monomer, gives the absorption shoulder from 610 to 620 nm. In this case, energy transfer in the trimer may be by Förster resonance from the α-84 PCB donor to the β-84 PCB acceptor (MacColl 2004; Loos et al. 2004).

4.1.4 Biosynthesis of Phycobiliproteins

Phycobiliproteins are a class of brilliantly colored proteins containing open-chain tetrapyrrole chromophoric groups apart from the apoprotein part. The biosynthesis of phycobiliproteins, viz., biosynthesis of tetrapyrrole phycobilins and their attachment to the apoproteins component are discussed here separately.

4.1.4.1 Biosynthesis of Phycobilins

Cyanobacteria can synthesize a vast array of tetrapyrroles like hemes, chlorophylls, phycobilins, siroheme, etc. As in case of most other organisms, δ-aminolevulinic acid (ALA) is the precursor of all tetrapyrroles in cyanobacteria. However, unlike animals and fungi, ALA in cyanobacteria (including all plants, algae, and most of the bacteria) is synthesized from glutamate (Glu). Glutamate is reduced to glutamate-1-semialdehyde (GSA) which supplies the C and N atoms of the tetrapyrrole nucleus. Asymmetric condensation of two molecules of ALA with the help of the enzyme porphobilinogen (PBG) synthase results in the formation of PBG. Uroporphyrinogen III (UPG III), the last common precursor of all end-product tetrapyrroles, is synthesized from PBG by two enzymes, viz., hydroxymethylbilane (HMB) synthase which converts PBG to HMB and uroporphyrinogen III synthase which catalyzes the formation of UPG III from HMB. UPG III can thus lead to the formation of protoporphyrin (precursor of chlorophylls, hemes, and phycobilins) or precorrins. Uroporphyrinogen decarboxylase enzyme decarboxylates all four acetate residues of UPG III to yield coproporphyrinogen (CPG). Another enzyme CPG oxidase oxidatively decarboxylates the propionate groups to produce protoporphyrinogen IX. This enzyme is specific for the III isomer of CPG over the nonphysiological I isomer. Protoporphyrinogen oxidase catalyzes the removal of six electrons from the tetrapyrrole macrocycle to form protoporphyrin IX which is the last biosynthetic step that is common to hemes, bilins, and chlorophylls (Fig. 4.1). Ferrochelatase catalyses the last step of protoheme formation: the insertion of Fe^{+2} into protoporphyrin IX. The increase in ferrochelatase activity is positively correlated with the accumulation which suggests that the level of this enzyme may be important rate-controlling factor in phycobilin synthesis (Brown et al. 1984). The opening of macrocycle by heme oxygenase enzyme results in conversion of protoheme to biliverdin IXα.

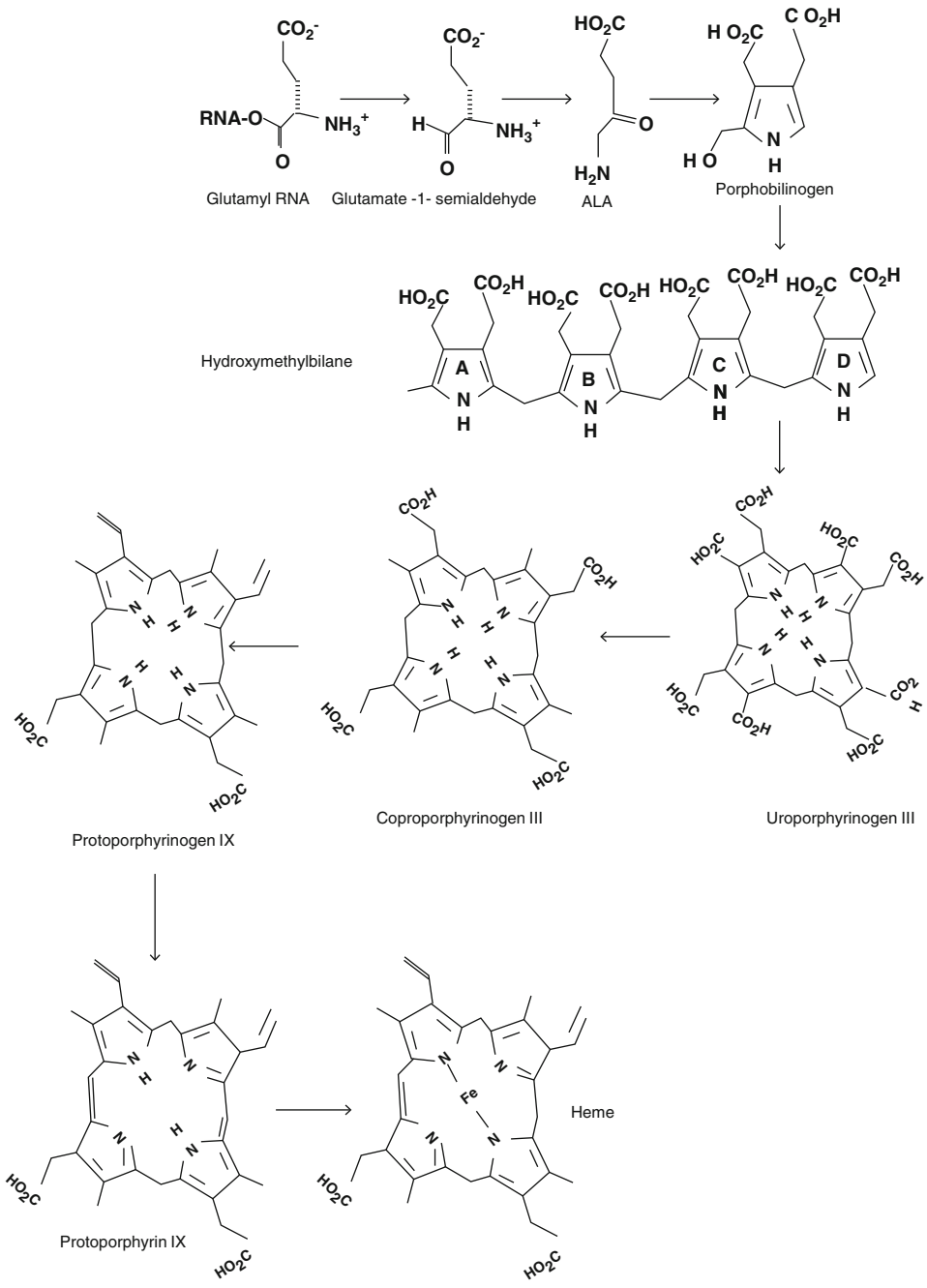

Fig. 4.1 Biosynthesis of heme from glutamate

Phycocyanobilin and phycoerythrobilin are isomers containing four more protons than their precursor, biliverdin. NADPH acts as electron donor for the reduction of biliverdin in the ferredoxin-linked reductase system (Beale and Cornejo 1991a). The reduction proceeds in two two-electron steps, with utilization of one NADPH molecule at each step (Beale and Weinstein 1990). Phycoerythrobilin is the first fully reduced bilin produced from biliverdin IXα

which is subsequently isomerized to phycocyanobilin (Beale and Cornejo 1991b). Phytochromobilin and 15, 16-dihydrobiliverdin are thought to be intermediates of these two two-electron reduction steps. It is proposed that phycobilin biosynthesis might proceed through phytochromobilin (Beale and Cornejo 1984). However, Beale and Cornejo (1991a, b) concluded that 15, 16-dihydrobiliverdin, rather than phytochromobilin, is the partially reduced intermediate in phycobilin synthesis (Fig. 4.2). Once the pyrrole ring is reduced, the vinyl group

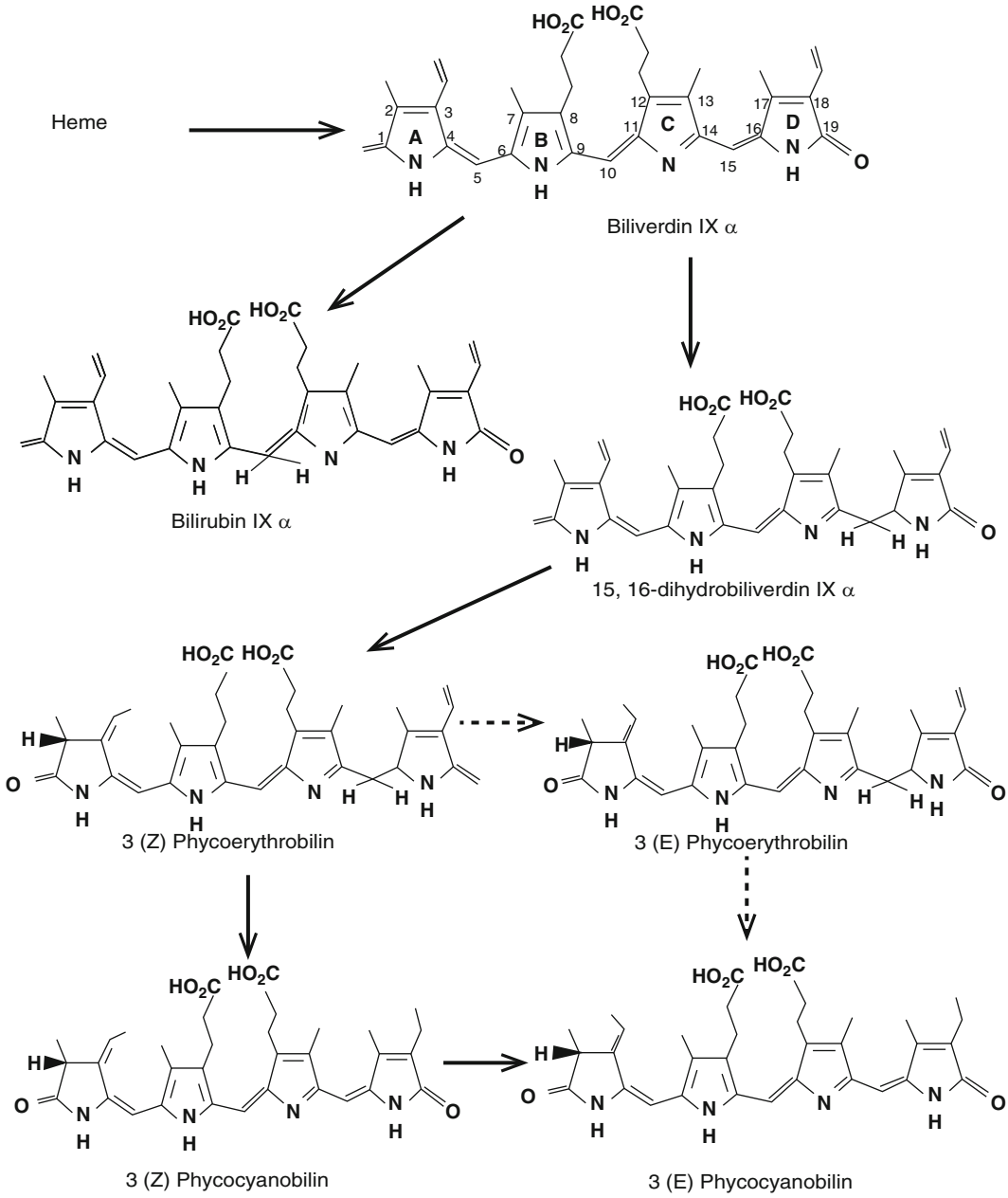

Fig. 4.2 Biosynthesis of phycobilins (Biliverdin IXα onwards) (Adopted from Schluchter and Glazer (1997). Originally referred by Schluchter and Glazer from Beale and Cornejo (1991a, b))

isomerizes to an ethylidene group to form 3(Z)-phycoerythrobilin. 3(Z)-phycocyanobilin dimethyl ester is the spontaneous isomerization product of synthetic 3-vinylphycocyanobilin dimethyl ester (Gossauer et al. 1989). The early phycobilin products are also formed with the ethylidene group in Z configuration. The 2-ethylidine isomer is reported to be the generally preferred initial product of vinyl isomerization although the E configuration is reported to be thermodynamically favored (Rüdiger et al. 1980; Weller and Gossauer 1980).

4.1.4.2 Ligation of Apoproteins to Phycobilins

Currently, there are four characteristic modes of chromophore attachment:

1. Spontaneous attachment: Many of previous studies reported that most apoproteins could bind phycobilins (phycocyanobilin, phycoerythrobilin) spontaneously in vitro; however, this process has quite low fidelity leading to mixed products (Arciero et al. 1988; Fairchild and Glazer 1994a; Schluchter and Glazer 1999). Spontaneous chromophore binding in vivo is unlikely as chromophores are reactive and present in very low concentration, and biliprotein synthesis is part of major metabolism in cyanobacteria.
2. Autocatalytic attachment: Autocatalytic attachment is a kind of spontaneous attachment leading to chromoproteins that are structurally and functionally indistinguishable from the respective native forms isolated from the parent organism (except for the lack of methylation of some β-subunits). Currently, correct chromophore binding, which is a true autocatalytic lyase activity, is mostly observed only among phytochromes (Wu and Lagarias 2000). Autocatalytic binding is also reported for ApcE, which could be reconstituted with phycocyanobilin (PCB) to give native-like $_{LCM}$ (Zhao et al. 2005).
3. E/F-type lyases: In *Synechococcus* PCC 7002, a heterodimeric lyase (CpcE/F) has been identified which catalyses both the forward (binding) and the reverse (releasing) reaction (Zhou et al. 1992; Fairchild et al. 1992). It also catalyses the addition of PEB to apoα-C-PC (CpcA), but with lower affinity as compared with PCB (Fairchild and Glazer 1994b). *pec* operons of *Nostoc* PCC7120 and *Mastigocladus laminosus* have been reported to have two homologous genes, *pecE/F* (Kufer et al.1991), coding for a variant of this lyase (Zhao et al. 2002). Phycourobilin (PUB) is another example of such a chromophore where D5-to-D2 double-bond isomerization generates bound PUB from PEB catalyzed by such isomerizing lyase that uses PEB as substrate. Evidence for such a lyase has been found in marine cyanobacteria like *Synechococcus* sp. WH81024. Cyanobacterial S/U-type lyases: High substrate specificity and small number of E/F-type lyases prompted the search for some other lyases in cyanobacteria. Few new types of lyases in *Synechococcus* sp. PCC7002 were reported by Shen et al. (2004). One of them, CpcS, is coded by a homologue of a gene, *cpeS*, which is reported in *Fremyella diplosiphon*, on an operon with *cpeR* associated with gene regulation (Cobley et al. 2002). The S/U type of lyases comprises a new family of proteins unrelated to the E/F-type lyases and exhibit large variations. The main feature of the S/U lyases is high specificity for the binding site along with a very low specificity for the chromophore and the receptor apoprotein. CpcS from *Nostoc* sp. PCC 7120 is a nearly universal lyase for PCB attachment at Cys-84 of apo-phycobiliproteins (Zhao et al.2007).

4.2 Organization and Regulation of Phycobiliprotein-Encoding Genes

Extensive structural and functional analyses of PBsome via molecular genetics have been performed for *Synechococcus* sp. strain 7002 (Bryant 1991) and for other cyanobacteria (Houmard et al. 1986; Grossman et al. 1995; Liu et al. 2005). Transcriptional units in different cyanobacteria

vary in numbers as well as in terms of distribution of genes. The organization of complete set of genes encoding structural components of PBsome for *Mastigocladus laminosus* is shown in Fig. 4.3.

The *cpc* operons are composed of genes encoding PBsome rod components and polypeptides involved in chromophore attachment to the PC^{α} subunit (*cpcE* and *cpcF* genes; Bryant 1988, 1991; Zhou et al.1992; Swanson et al. 1992; Fairchild et al. 1992). The *cpcG* gene(s), encoding the L_{RC}, is located on *pec-cpc* super operon in *Anabaena* sp. strain PCC 7120 and *Mastigocladus laminosus*, whereas the same gene is reported to be on other transcriptional unit in *Synechococcus* sp. strain PCC 7002. Liu et al. (2005) reported a *cpc* operon from *Arthrospira platensis* FAHCB341 which contained six parts: the 427 bp upstream sequence (*ussB*), 519 bp *cpcB* gene, 111 bp intergenic spacer between *cpcB* and *cpcA* genes, 489 bp *cpcA* gene, 184 bp upstream sequence (*ussH*) of *cpcH*, and partial sequence of *cpcH* gene (357 bp) which related to the synthesis of one of the rod-linker polypeptide. In all cyanobacteria studied till date, the *cpcA* gene coding for the α subunit of PC is located downstream from the *cpcB* gene encoding β subunit of PC. In some cyanobacteria, multiple gene sets for some types of phycobiliproteins and linker polypeptides have been found. For example, two *cpcBA* gene sets, viz., *cpcB1A1* and *cpcB2A2* occur in *Synechococcus* sp. strain PCC 6301 (Lind et al. 1987; Lau et al. 1987; Kalla et al. 1988) and *Pseudanabaena* sp. strain PCC 7409 (Dubbs and Bryant 1987, 1993), while three different *cpcBA* gene sets have been reported in *Calothrix* sp. strain PCC 7601 (Mazel et al. 1988; Capuano et al. 1988; Mazel and Marliere 1989). In *Synechococcus* sp. strain PCC 6301 and PCC 7942, the duplicated phycocyanin genes encode identical polypeptides and are arranged as a tandem repeat unit with the genes for the rod linkers between the *cpcB1A1* gene set and the downstream *cpcB2A2EF* gene set (Kalla et al. 1988, 1989).

The *cpeBA* operon consisting of the genes encoding for PE has the same order of genes, i.e., *cpeB* gene is located upstream to the *cpeA* gene (Mazel et al. 1986; Dubbs and Bryant 1987, 1993; Anderson and Grossman 1990; Bernard et al. 1992). In *Calothrix* sp. strain PCC 7601, the two genes, *cpeB* and *cpeA*, were found to be separated by 79 base pairs (Mazel et al. 1986). A similar orientation is also reported in *Pseudanabaena* sp. PCC 7409 where the two genes were separated by 74 base pairs (Dubbs

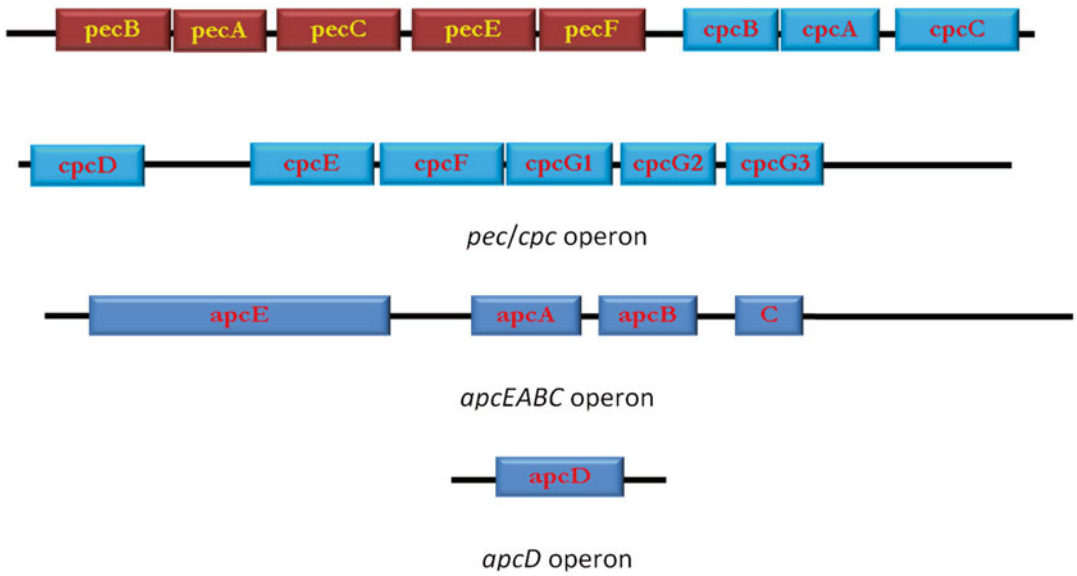

Fig. 4.3 Organization of phycobiliprotein genes in *Mastigocladus laminosus* (Adopted from Bhaya et al. (2000))

and Bryant 1987, 1993). However, none of these two cyanobacteria contained genes for linker polypeptides in *cpe*BA operon. In *Calothrix* sp. strain 7601, *cpeCDE* operon contained the genes for PE-associated rod-linker polypeptides that are only transcribed when cells are grown in green light (Federspiel and Grossman 1990; Federspiel and Scott 1992). DNA sequence analysis of *Synechococcus* sp. strain WH 8020 showed the genes for the α and β subunits of PE-I and PE-II as well as for the γ subunit of PE-II to be clustered in a large 15 kb region of the genome. Twelve open reading frames are included in this region (Wilbanks and Glazer 1993).

Unlike *cpc* and *cpe* operons, the *apc* operons have a reverse order of the genes: the *apcA* gene is located upstream to the *apcB* gene (Bryant 1988, 1991). The *apc* operons from several cyanobacteria like *Synechococcus* sp. strain PCC 6301 (Houmard et al. 1986), *Synechococcus* sp. PCC 7002 (Bryant 1988, 1991), *Calothrix* sp. strain 7601 (Houmard et al. 1988, 1990), *Anabaena variabilis* (Johnson et al. 1988), etc. The *apcC* gene encoding the small linker polypeptide $L_C^{8.9}$ lies downstream from the *apcB* gene. The *apcD* and *apcF* genes encode minor APC-related phycobiliproteins. The *apcE* encodes the large core membrane linker phycobiliprotein (L_{CM}). In *Synechococcus* sp. strain PCC 7002 (Bryant 1988, 1991), the *apcE* gene is transcribed as a separate unit, whereas in *Synechococcus* sp. strain PCC 6301 (Capuano et al. 1991), *Calothrix* sp. strain 7601 (Houmard et al. 1990), and *Mastigocladus laminosus*, it is located on a transcriptional unit with *apcA*, *apcB*, and *apcC*.

As cyanobacteria can be found in locations with varying chemical and physical parameters like nutrient availability, quality and quantity of light, temperature, water activity, etc., the expression of phycobiliprotein coding genes also modulate accordingly to adapt to particular conditions. Bryant (1981) studied the regulation of PC synthesis in response to growth in chromatic illumination in 69 cyanobacteria and reported controlled phycocyanin synthesis through photoregulated expression of the α and β subunits. For these strains, the expression of one pair of PC subunits was constitutive irrespective of light quality while expression of the other occurred particularly under red light. Oelmuller et al. (1988) showed that the levels of transcripts encoding the APC subunits, the core linker polypeptide, and the constitutive PC subunits were similar under red and green light conditions while very large increases PE and inducible PC mRNAs occurred rapidly after transferring *Fremyella diplosiphon* from red light to green light and green light to red light, respectively. The green light condition used in those experiments was effective in eliminating essentially all inducible PC mRNA synthesis while the red light did not eliminate all PE mRNA synthesis and the decrease in the phycoerythrin mRNA level in red light was much slower than the decline in the levels of the inducible phycocyanin transcripts in green light. Grossman et al. (1988) reported that mRNA of *cpc1* operon in *Calothrix* sp. strain PCC 7601 accumulated constitutively while the transcript of *cpc2* operon accumulated only when the cells are grown in red light. Similar results have been described for the group III chromatic adapter *Pseudanabaena* sp. strain PCC 7409 (Dubbs and Bryant 1993).

Fremyella diplosiphon UTEX481 (*Calothrix* sp. strain PCC 7601) is widely studied for changes in its PBP composition in response to varying light color. Studies have shown that the quality of light could modulate the composition of PBsome through a couple of two-component regulatory system. The regulator for complementary chromatic adaptation (Rca) activates *cpc2* and *pcyA* and represses *cpeC* expression in red light, while the control of green light induction (Cgi) system has no detectable effect on *cpc2* or *pcyA* expression and only represses *cpeC* expression in red light (Sobczyk et al. 1994; Li et al.2008; Seib and Kehoe 2002; Alvey et al. 2003; Li and Kehoe 2005; Kehoe and Gutu 2006). The final gene in the *cpeC* operon, *cpeR*, encodes an activator required for *cpeBA* and *pebAB* expression (Cobley et al. 2002; Seib and Kehoe 2002). Both the Rca and Cgi systems regulate these two operons by controlling *cpeC* expression.

Light intensity is the most significant environmental factor influencing the light harvesting complexes (phycobilisomes) in cyanobacteria. The influence of light intensity on PBP content

has been reported in different organisms (Yamanaka and Glazer 1980; Lonneborg et al. 1985; Muller et al. 1993; Gamier et al. 1994). de Lorimier et al. (1992) reported 1.8-fold decrease in PC:APC ratio in *Agmenellum quadruplicatum* PR-6 during growth in a light intensity of 1260 μE as compared to that in 20 μE. The relative level of RNAs encoding phycocyanin and allophycocyanin was found to vary with light intensity in parallel with the phycobiliprotein ratio. Nomsawai et al. (1999) reported that rapid changes in light intensity from high to low light caused a dramatic increase in the accumulation of PC, APC, and Chl *a*. *Spirulina platensis* Cl underwent a significant change in pigmentation mainly due to the reduction of PC and Chl *a* synthesis under high light intensity. Shifting cells from high-light to low-light conditions resulted from an increase in transcription levels of the *cpc* and *apc* operons ultimately increasing the content of PBPs. When cells were shifted from low to high light, the 3.5 kb transcript species corresponding to the *cpcBAHID* operon disappeared in concomitant with the disappearance of 33 kDa linker polypeptide within 24 h. However, the other two rod-linker polypeptides, 34.5 and 15 kDa, were still associated with the intact phycobilisomes.

Hihara et al. (2001) reported downregulation of *cpc* genes in *Synechocystis* sp. PCC 6803 grown under high light intensity (300 μmole photons/m^2/s) and *cpc* genes were downregulated more strictly than *apc* genes because phycocyanin is the primary target for the reduction of antenna size. It was interesting to note that *cpcD*, which is part of the *cpcBACD* operon, apparently was not downregulated as much as the other *cpc* genes. Singh et al. (2008) also reported downregulation of phycocyanin-encoding genes in *Synechocystis*.

Upon transfer to high light intensity conditions, both the amounts of photosystems and PBsomes are downregulated to avoid absorption of excess light energy. The capacity for CO_2 fixation and other cellular metabolism is upregulated to increase energy consumption. Protection mechanisms to prevent photodamage have been developed to cope with the increased production of reactive oxygen species. However, the molecular mechanisms that enable the cells to perceive and acclimate to the changing light environment were poorly understood at that time (Muramatsu and Hihara 2012).

As mentioned earlier, phycobiliprotein synthesis as well expression of the phycobiliprotein-encoding genes may also be modulated by nutrients like nitrogen, sulfur, and iron. Iron-, nitrogen-, and sulfur-deficient cells contain less than half of the normal complement of thylakoid membranes (Sherman and Sherman 1983; Wanner et al. 1986). The remaining membranes are disorganized and interspersed with large deposits of glycogen. A dramatic response of cyanobacteria to nutrient limitation is a decrease in the abundance of pigment molecules in the cell. Cultures deprived of nitrogen for 30 h exhibited no detectable PC and sulfur-deprived cultures showed a similar rapid decline in the absolute levels of PC and APC (Collier and Grossman 1992; Wanner et al. 1986). During sulfur and nitrogen starvation, there was a rapid and near complete degradation of the PBsome. PBsome degradation could provide nitrogen-limited cells with amino acids used for the synthesis of proteins important for the acclimation process. It is more difficult to understand why PBsome would be degraded in cells limited for other macronutrients, such as sulfur, since phycobiliproteins are a poor source of sulfur amino acids. However, the PBsome was degraded in the same ordered manner in both sulfur- and nitrogen-starved cells (Collier and Grossman 1992; Yamanaka and Glazer 1980). The levels of mRNAs encoding the phycobiliproteins have also been reported to decline during nutrient-limited growth. de Lorimier et al. (1984) reported that the level of PC mRNA decreased to nearly zero in nitrogen-starved *Synechococcus* sp. strain PCC 7002. Further analyses had shown that 3–5 h after the initiation of nitrogen deprivation, the transcripts from the *cpcBA* operon were essentially undetectable (Bryant 1991). The results obtained by Gasparich et al. (1987) using *cpcB-lacZ* fusion construct suggested that nitrogen deprivation resulted in a marked decrease in the transcription of *cpcBA*. In *Synechococcus* sp. strain PCC 7942, the mRNAs encoding both PC and APC declined rapidly during nitrogen or

sulfur limitation and less rapidly during phosphorus limitation. However, levels of all of these mRNA species remained at 5–10 % that of nutrient-replete cells (Collier and Grossman 1992), even 48 h after the cells were transferred to medium lacking nitrogen or sulfur. This change in the steady-state levels of phycobiliprotein mRNAs may be a consequence of both altered rates of transcription and mRNA turnover. These results suggested that the production of phycobiliproteins during nutrient-limited growth might be blocked by both transcriptional and posttranscriptional events.

Similarly, iron has been shown to modulate PBP production and regulation. Hardie et al. (1983) reported that C-PC content in *Agmenellum quadruplicatum* started decreasing after 16 h and decreased up to 200 h until iron was replenished in the medium. Troxler et al. (1989) showed that phycobiliprotein mRNA, which was absent in dark-grown cultures of *Cyanidium caldarium*, appeared when the culture was grown in presence of heme (Fe-protoporphyrin IX) under dark conditions. This was the first report to show that heme is regulatory factor specifically involved in transcriptional regulation of phycobiliprotein genes. Singh et al. (2003) reported downregulation of *cpc* genes in *Synechocystis* sp. strain PCC 6803 when the cyanobacteria was grown in iron-deficient BG-11 (with nitrogen) medium. Hemalata and Fatma (2009) observed that phycobiliprotein content in *Anabaena* NCCU 9 decreased significantly when grown in iron-deficient medium. Chakdar (2012) reported that ferric ammonium citrate (1.2 ppm) was optimal iron source for maximal phycocyanin production in three heterocystous cyanobacteria. In the same study, modulation of *cpcB* gene was also reported by concentration and source of iron which was probably modulated through altered synthesis of heme or phycocyanobilins (Fig. 4.4).

The composition and function of PBPs in cyanobacteria have been reported to change in response to stress conditions (Grossman et al. 1993), but among various stresses, acclimation to high salt concentrations is of high importance for basic as well as applied research, since a high percentage of irrigated land suffers from increasing levels of salts. Marin et al. (2004) reported that *cpcBACD* genes were downregulated in *Synechocystis* sp. strain 6803 when the cyanobacteria was grown with 684 mM NaCl. Chakdar et al. (2012) also reported modulation of cpcB gene under salinity. It was observed that cpcB gene expression and phycocyanin production was upregulated when *Anabaena* (CCC162 & CCC421) and *Nostoc* (CCC391) were grown with 10 mM Nacl. Rapid entry of sodium ions might result in detachment of phycobilisomes from the thylakoid membranes, resulting in decreased photosynthesis (Rafiqul et al. 2003), energy transfers from phycobiliproteins to PS II reaction center (Schubert et al. 1993; Verma and Mohanty 2000), and uptake of other mineral nutrients, such as K^+, Ca^{2+}, and Mn^{2+} (Hasegawa et al. 2000).

4.3 Environmental Stress Conditions for Enhancing Phycobiliprotein Production

As cyanobacteria can be found in locations which exhibit widely fluctuating chemical and physical parameters like nutrient availability, light intensity, light wavelength, temperature, water activity, etc., synthesis of phycobiliprotein is also accordingly modulated to adapt to a particular condition. The composition and function of phycobiliproteins in cyanobacteria have also been reported to change under stress conditions (Grossman et al. 1993). Among various conditions which influence the phycobiliprotein production, light, temperature, and pH are most important. Appropriate alteration of such parameters can render enhanced production of phycobiliproteins.

4.3.1 Light

Both color and intensity of light can influence phycobiliprotein synthesis in cyanobacteria. Complementary chromatic adaptation (CCA) has been a well-studied phenomenon in cyanobacteria where the relative amount of PC and PE

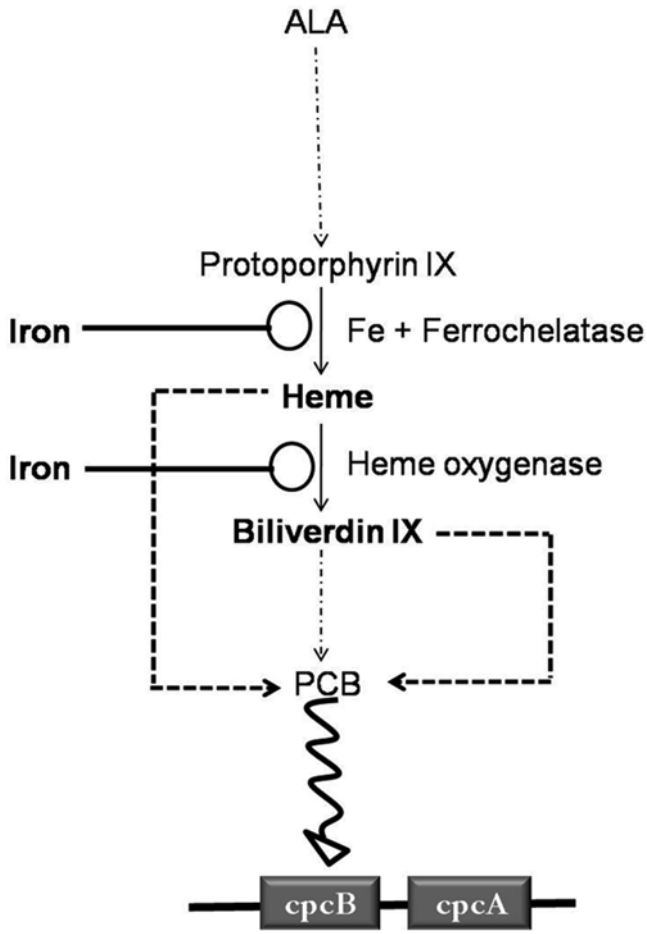

Fig. 4.4 Probable mode of regulation of cpcB gene through iron- A hypothetical view ——○ indicates steps where probably the key enzymes in PCB biosynthesis are regulated by iron which ultimately influenced the synthesis of Heme or Biliverdin IX; ---------- indicates the regulation by the key products like heme or biliverdin IX which influences the synthesis of PCB. This could be the possible way through which iron actually regulates the expression of phycocyanin beta subunit gene (Adapted from Chakdar 2012)

changes depending on light color. CCA has been most extensively studied in the cyanobacterium *Calothrix* sp. strain 7601 (also known as *Fremyella diplosiphon*), although the phenomenon has been observed in a number of other cyanobacteria (Tandeau de Marsac 1977; Bryant and Cohen-Bazire 1981; Bryant 1981, 1982). Stowe et al. (2011) showed that *F. diplosiphon* exhibited a 3.3-fold induction of PC in red light and a 5.77-fold induction of PE in green light. In the same study, they reported that, in *Gloeotrichia* UTEX 583, PE was synthesized in green light and not synthesized in red light, but PC is highly abundant in both red and green light. So, chromatic adaptation property of cyanobacteria can be exploited for commercial production of high-value pigments like phycocyanin and phycoerythrin.

Light intensity is the most significant environmental factor influencing the light-harvesting complexes (phycobilisomes) in cyanobacteria. As such, light intensity is of particular importance to the outdoor cultivation since it is largely variable in natural environment. The influence of light intensity on PBP content has been reported in different organisms (Yamanaka and Glazer

1980; Lonneborg et al.1985; Muller et al.1993; Gamier et al. 1994).

de Lorimier et al. (1992) reported 1.8-fold decrease in PC:APC ratio in *Agmenellum quadruplicatum* PR-6 during growth in a light intensity of 1260 µE as compared to that in 20 µE. Hemalata and Fatma (2009) reported that 25 µmol photons/m^2/s to be the most suitable light intensity for maximum production (124.95 mg/g dry wt.) of phycocyanin in *Anabaena* NCCU9. 25 µmol photons/m^2/s was also reported to be optimum light intensity for *Synechococcus* NKBG 042902 (Takano et al. 1995), *Spirulina subsalsa*, *S. maxima* (Tomasseli et al. 1995, 1997), and *Synechocystis* (Hong and Lee 2008), while 12.5 µmol photons/m^2/s was found optimum for *Nostoc* UAM 206 (Poza-Carrion et al. 2001) and *N. muscorum* (Ranjitha and Kaushik 2005a). On the other hand, 150 µmol photons/m^2/s was optimal light intensity for phycobiliprotein production in *Arthronema africanum* (Chaneva et al. 2007). However, a number of studies have reported reduction in phycobiliprotein gene expression and synthesis (Hihara et al. 2001; Tu et al. 2004). Upon transfer to high light conditions, both the amounts of photosystems and the light-harvesting antenna complex, i.e., phycobilisome (PBsome), are downregulated to avoid absorption of excess light energy (Muramatsu and Hihara 2012). In fact, when the amount of light energy absorbed by light-harvesting complexes exceeds the limit of energy requirements by various cellular processes, the excess energy produces reactive oxygen species (ROS) leading to severe photodamage to cellular components. Nonetheless, photosynthetic organisms have evolved different acclimatory responses to high light conditions to maintain a balance between supply and consumption of energy and to protect the photosynthetic apparatus from damage. Cyanobacteria prefer low light intensities and stimulate phycobiliprotein synthesis due to their low specific maintenance energy and their pigment composition. Not only this, low irradiances actually broadens the overall light absorption band in such a way that the balance of light energy distribution between the two photosystems is maintained that optimizes the rate of light energy conversion.

4.3.2 Temperature

Like any other organism, all metabolic processes in cyanobacteria are also influenced by temperature. The optimal growth temperature and tolerance to the extreme values vary from strain to strain. Phycobiliprotein synthesis in cyanobacteria is also regulated by temperature, and depending on the strain, the optimum temperature for maximum production of phycobiliprotein production varies. Anderson et al. (1983) reported that PE to PC ratio in *Nostoc* sp. strain MAC increased when the cyanobacteria was grown at 39 °C (PE:PC = 2.3:1) as compared to growth at 30 °C (PE:PC = 1:1) under white light and light intensity of 10^{-2} µE/cm^2s. Like light quality and quantity, temperature can also regulate the synthesis of phycobiliproteins and change the relative amount of PC and PE according to the conditions. Hemalata and Fatma (2009) showed that a temperature of 30 °C is optimum for maximum production (127.02 mg/g of dry wt.) of phycobiliproteins in *Anabaena* NCCU9, and phycobiliprotein production reduced significantly at higher or lower temperature (23.6 % reduction at 20 °C and 38 % reduction at 40 °C). Chaneva et al. (2007) reported that phycobiliprotein content in *Arthronema africanum* increased up to 36 °C under a light intensity of 150 µmol photons/m^2/s and correlated to the changes in growth curve. But further increase in temperature resulted in decrease in phycobiliprotein content by the tune of 10–20 %. Sakamoto and Bryant (1998) reported a temperature of 36 °C to be most suitable for maximum production of phycobiliproteins in *Synechococcus*. In *Plectonema boryanum* UTEX 485, phycobiliprotein pigments reduced significantly at 15 °C as compared to 29 °C temperature under light intensity of 150 µmol photons/m^2/s (Misckiewicz et al. 2002). Most of these studies (Chaneva et al. 2007; Misckiewicz et al. 2000) were carried out to assess the combined effect of light intensity and temperature on phycobiliprotein production in different cyano-

bacteria, and it was clear from these studies that influence of light intensity and temperature in combination is more pronounced than the individual effects of these two factors. So, optimization of both the parameters rather than one of the factors will be the appropriate intervention for enhancing the production of phycobiliprotein production in cyanobacteria.

4.3.3 pH

In general, cyanobacteria are alkalophiles capable of maintaining an internal, constant pH of 7.1–7.5 in the range of external pH from 5 to 10 (Ritchie 1991). Changes in pH affect the solubility and bioavailability of nutrients, transport of substances across the cytoplasmic membranes, and the activity of intra- and extracellular enzymes, as well as photosynthetic electron transport and the osmotic potential of the cytoplasm (Walsby 1982). It was reported that phycobiliprotein concentration in *Gloeotrichia natans* changed significantly with respect to changes in the pH of the medium; however, growth rate of the cyanobacterium remained unaffected (Boussiba 1991). At a pH of 7.00, phycobiliprotein constituted up to 10 % of the total protein, but at pH 9.0, the amount was 28 % of the total protein. In *Nostoc* sp. UAM206, allophycocyanin content increased significantly at pH 9.0 as compared to pH 7.0 (Poza-Carrion et al. 2001). *Nostoc* sp. UAM206 exhibited an increase in PC and PE with the increase in pH under limited availability of inorganic carbon; however, when inorganic carbon was made available, the effect of pH was nullified (Poza-Carrion et al. 2001). Hence, the effect of pH on photosynthetic pigments is more pronounced under nutrient-limited conditions. Desmukh and Puranik (2012) also reported pH 10.0 to be the optimum pH for phycobiliprotein production in *Synechocystis* sp., while Hong and Lee (2008) showed pH 8.0 to be best for phycobiliprotein production in *Synechocystis* sp. PCC 6701. *Anabaena* NCCU9 also resulted in maximum phycobiliprotein production (102.24 mg/g dry wt.) when grown at pH 8.0 but the culture became white at pH 2 and 12 (Hemalata and Fatma 2009). For a particular culture, it is always essential to determine the optimum pH of the medium in which the culture can perform the best in terms of phycobiliprotein production.

4.3.4 Salt Stress

Among various stress conditions, salt stress is of utmost importance for basic as well as applied research, since a high percentage of irrigated land suffers from increasing levels of salts. Salt stress mainly results in decrease of phycocyanin content and thereby interrupts the energy transfer from phycobiliproteins to PSII reaction center (Schubert and Hagemann 1990; Schubert et al. 1993; Lu et al. 1999; Lu and Vonshak 2002). However, there has been no study to point out if there is any specific effect of salt stress on phycocyanin production as well as the expression of phycocyanin-encoding genes. Marin et al. (2004) in order to study genome-wide response of gene expression under salt stress, reported that *cpcBACD* genes were downregulated in *Synechocystis* sp. strain 6803 when the cyanobacteria was grown with 684 mM NaCl. Phycocyanin production as well as relative expression of *cpcB* gene increased in *Nostoc commune* (CCC391) and *Anabaena variabilis* (CCC421) when grown with 10 mM NaCl (Chakdar 2012). Hemalata and Fatma (2009) also reported enhancement of phycobiliprotein production in *Anabaena* NCCU9 with 10 mM sodium chloride. Although, all cyanobacteria do not respond in a similar fashion to salt stress, it can be used as a stimulus for salt-tolerant cyanobacteria to produce higher amount of phycobiliprotein

4.4 Extraction and Purification Strategies for Phycocyanin and Phycoerythrin

Commercial exploitation of phycobiliproteins requires rapid and efficient extraction followed by effective separation from undesirable proteins using appropriate purification strategy. But,

extraction of phycobiliproteins from cyanobacteria is notoriously difficult because of their extremely resistant multilayered cell wall and considerably small size (Stewart and Farmer 1984; Wyman 1992). A number of methods have been employed to extract and quantify phycobiliproteins from different cyanobacteria; however, there is no standard technique for quantitative extraction of pigments from these organisms (Jeffrey and Mantoura 1997; Wiltshire et al. 2000). Methods like freezing and thawing (Abalde et al. 1998; Minkova et al. 2003), use of sonicators (Abalde et al. 1998) and French pressure cells (Alberte et al. 1984), use of enzymes like lysozyme (Boussiba and Richmond 1979), combination of EDTA and lysozyme (Stewart and Farmer 1984; Kilpatrick 1985; Vernet et al. 1990), etc., have been employed for disruption of the cells in order to release phycobiliproteins. Viskari and Colyer (2003) described a method for extracting phycobiliproteins from a *Synechococcus* CCMP 833 cyanobacteria culture that utilizes 3 % 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) 0.3 % asolectin combined with nitrogen cavitation and achieved extraction efficiencies of greater than 85 %. Lawrenz et al. (2011) reported that disruption of cells by freezing–thawing and sonication both resulted in significantly higher extraction efficiencies than disruption with a tissue grinder. It has been reported by other workers (Abalde et al. 1998; Doke 2005) also that freeze/thaw cycles was the most efficient and economical way to extract C-PC from wet cyanobacterial biomass. Different buffers were generally used to extract phycobiliproteins with reliance on spectrometric detection and quantification. Phosphate buffer is mostly used for such extraction (Sarada et al. 1999; Doke 2005; Ranjitha and Kaushik 2005b; Benedetti et al. 2006); however, some people have also used Tris buffer (Bhaskar et al. 2005), HEPES buffer (Gupta and Sainis 2009), etc.

A number of processes are available for purification of PC and PE from cyanobacteria. Most of these procedures involve steps like ammonium sulfate precipitation, dialysis, gel filtration chromatography, anion exchange chromatography, hydroxy apatite chromatography, etc. A few procedures have also been reported which involve some additional steps like membrane filtration or use of organic solvents. These purification strategies involving a different combination of the abovementioned steps have been employed to purify PC and PE from a number of cyanobacteria. Some of the strategies used for purification of PC from cyanobacteria are presented in Table 4.1 (modified from Eriksen 2008). Abalde et al. (1998) reported 83.4 % recovery with a purity of 3.2 from *Synechococcus* sp. IO920 after hydrophobic interaction chromatography, while Santiago-Santos et al. (2004) obtained 80 % recovery with a purity ratio of 3.5 using similar procedure. Abalde et al. (1998) reported a final purity of 4.85 with 76.56 % recovery following a combination of hydrophobic interaction chromatography (using butyl sepharose) and ion exchange chromatography (using Q Sepharose), while Boussiba and Richmond (1979) attained a purity of 4.15 in *S. platensis* following hydroxyapatite and ion exchange chromatography. Following a novel method using rivanol treatment, 45.7 % PC with a purity of 4.30 was recovered from *S. fusiformis* (Minkova et al. 2003). In 2006, Benedetti et al. reported a purity of 4.78 from *Aphanizomenon flos-aquae* after hydroxyapatite chromatography. Soni et al. (2006) used ammonium sulfate precipitation, gel filtration chromatography, and DEAE-cellulose column chromatography to obtain phycocyanin with a purity of 3.31 from *Oscillatoria quadripunctulata* while they further attained PC with a purity of 4.52 from *Phormidium fragile* using a novel single-step hydrophobic interaction chromatography (Soni et al. 2008). Recently, Yan et al. (2011) attained a purity of 5.59 with a final PC recovery of 67.04 % from *Spirulina platensis* following ammonium sulfate precipitation and anion exchange chromatography using DEAE sepharose fast flow column. Patil et al. (2006) studied purification of C-PC by aqueous two-phase extraction (ATPE) combination with ion exchange chromatography, and the results showed that ATPE purification yielded high-purity C-PC with 73 % recovery. This not only enabled an increase in product purity without the

Table 4.1 Strategies used for purification of phycocyanin from different cyanobacteria

Numbers and types of central unit operations	Cyanobacterial species	Reference
Chromatography based methods		
1. Ammonium sulfate precipitation 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	*Spirulina platensis*	Boussiba and Richmond (1979)
1. Hydrophobic interaction chromatography 2. Ion exchange chromatography	*Synechococcus* sp.	Abalde et al. (1998)
1. Ammonium sulfate fractionation 2. Ion exchange chromatography 3. Gel filtration	*S. platensis*	Zhang and Chen (1999)
1. Rivanol treatment 2. Ammonium sulfate precipitation 3. Gel filtration 4. Ammonium sulfate precipitation	*S. fusiformis*	Minkova et al. (2003)
1. Ammonium sulfate precipitation 2. Size exclusion chromatography 3. Anion exchange chromatography	*Oscillatoria quadripunctulata*	Soni et al. (2006)
1. Ammonium sulfate precipitation 2. Hydroxyapatite chromatography	*Aphanizomenon flos-aquae*	Benedetti et al. (2006)
1. Expanded bed adsorption chromatography 2. Anion exchange chromatography	*S. platensis*	Niu et al. (2007)
1. Ammonium sulfate fractionation 2. Hydrophobic interaction chromatography	*Phormidium fragile*	Soni et al. (2008)
1. Ammonium sulfate precipitation 2. Dialysis 3. Anion exchange chromatography	*S. platensis*	Yan et al. (2011)
1. Ammonium sulfate precipitation 2. Dialysis 3. Anion exchange chromatography	*Nostoc commune*, *Anabaena oryzae*, *and A. variabilis*	Chakdar (2012)
Two phase aqueous extraction based methods		
1. Two-phase aqueous extraction 2. Ultrafiltration 3. Ammonium sulfate precipitation	*S. maxima*	Rito-Palomares et al. (2001)
1. Chitosan adsorption 2. Two-phase aqueous extraction	*S. platensis*	Patil et al. (2006)
1. Chitosan adsorption 2. Two-phase aqueous extraction 3. Ion exchange chromatography	*S. platensis*	Patil et al. (2006)
1. Repeated two-phase aqueous extraction 2. Ultrafiltration	*S. platensis*	Patil and Raghavarao (2007)

need of multiple steps but also reduced the volume of the contaminant proteins.

The basic strategies for purification of PE are almost same as for PC. Although most of the PE purification studies have been carried out in eukaryotic alga, some reports are also available for cyanobacteria. Tchernov et al. (1999) achieved a purity of more than 5 from *Nostoc* sp. following rivanol treatment, gel filtration chromatography, aminohexyl-sepharose column chromatography, and ammonium sulfate precipitation. Reis et al. (1998) obtained a purity of 5 from *Nostoc* sp. after ammonium sulfate precipitation, gel filtration chromatography, and ion

exchange chromatography using Q-sepharose fast flow column. Eighty-five percent recovery of phycoerythrin with a purity of 2.89 after 55 % ammonium sulfate precipitation from *Nostoc muscorum* has been reported (Ranjitha and Kaushik 2005b), while 72 % PE was recovered with a purity of 8.12 following anion exchange chromatography. Tripathi et al. (2007) also reported a purity of 5.25 from *Lyngbya arboricola* using a procedure involving acetone precipitation, gel filtration in addition to ammonium sulfate precipitation, and DEAE-cellulose column chromatography. Eighty percent recovery of PE content with a purity ratio of around 1.5 for young and old cultures from three cyanobacteria, viz., *Phormidium* sp. A27DM, *Lyngbya* sp. A09DM, and *Halomicronema* sp. A32DM, has been reported after the treatment of crude extract with 70 % ammonium sulfate (Parmar et al.2011). Fractionation of this PE-enriched 70 % ammonium sulfate precipitate of the crude extract with Sephadex G-150 further increased the purity ratio of PE to 3.9 and 3.6 for young and old culture of *Phormidium* sp. A27DM, 3.7 and 3.6 for young and old culture of *Lyngbya* sp. A09DM, and 4.0 for both the young and old culture of *Halomicronema* sp. A32DM respectively (Parmar et al.2011). The yield of PE obtained by the described extraction procedure was 62.6 %, 64.9 %, and 66.2 % for young cultures of *Phormidium* sp. A27DM, *Lyngbya* sp. A09DM, and *Halomicronema* sp. A32DM, respectively, and 68.2 %, 74.0 %, and 68.2 % for old cultures of *Phormidium* sp. A27DM, *Lyngbya* sp. A09DM, and *Halomicronema* sp. A32DM, respectively (Parmar et al. 2011). Chakdar and Pabbi (2012) developed a simple protocol for purifying phycoerythrin (PE) from *Anabaena variabilis* (CCC421) involving ammonium sulfate precipitation and dialysis followed by a single-step anion exchange chromatography using DEAE-cellulose-11 and acetate buffer. Precipitation of phycobiliproteins with 65 % ammonium sulfate resulted in 85.81 % recovery of PE with a purity of 2.81, while 62.5 % PE was recovered after chromatographic separation with a purity of 4.95.

4.5 Applications of Phycobiliproteins

Phycobiliproteins (PBPs) as natural colorants are gaining importance over synthetic colors as they are environment friendly, nontoxic, and noncarcinogenic. Dainippon Ink and Chemicals (Sakura, Japan) has developed a product called "Lina blue" (PC extract from *S. platensis*) which is used in chewing gum, ice sherbets, popsicles, candies, soft drinks, dairy products, and wasabi. Despite its lower stability to heat and light, phycocyanin is considered more versatile than gardenia and indigo, showing a bright blue color in jelly gum and coated soft candies (Lone et al. 2005). Besides this, there are number of other companies commercializing different products based on phycocyanins like C-Phycocyain from Cyanotech; PhycoLink® Biotinylated C-Phycocyanin from PROzyme; PhycoPro™ C-Phycocyanin from Europa Bioproducts Ltd.; C-Phycocyanin from Sigma Aldrich, C-Phycocyani from Fisher Scientific; etc. (Chakdar et al.2012). Use of phycobilins in cosmetics like lipstick, eyeliners, etc., is also gaining momentum. Properties like high molar absorbance coefficients, high fluorescence quantum yield, large Stokes shift, high oligomer stability, and high photostability make PBPs particularly PC and PE very powerful and highly sensitive fluorescent reagents. Purified native phycobiliproteins and their subunits fluoresce strongly and have been widely used as external labels for cell sorting and analysis and a wide range of other fluorescence based assays (Glazer and Stryer 1984). A number of commercial products based on C-phycoerythrin are also available in the market like Lightning Link® C-PE from Innova Biosciences Ltd., C-Phycoerythrin and Lightning Link C-PE antibody labeling kit from Novus Biologicals, Monoclonal Anti-mouse-Junctional Adhesion Molecule-C-Phycoerythrin from R & D Systems Inc., C-Phycoerythrin from Hash Biotech Ltd., Biotin Cr-PE (C-Phycoerythrin) from AssayPro, Lightning Link C-Phycoerythrin from Gentaur Molecular Products, and Stretavidin C-Phycoerythrin from Sigma Aldrich. The stabi-

lized phycobilisomes designated PBXL-3 L was accessed as a fluorochrome for flow cytometric immuno-detection of surface antigens on immune cells (Telford et al. 2001). PE is also a very important reagent in proteomics and genomics and form the basis of the detection system in Affymetrix chips (DNA microarrays). Phycoerythrin-labeled streptavidin is added after complete binding and produces a strong signal from array elements containing the biotin-labeled DNA or protein probes (De Rosa et al. 2003). In vivo fluorescence from PC has been used for online monitoring of growth in cyanobacterial cultures (Sode et al. 1991), detection of toxic cyanobacteria in drinking water (Izydorczyk et al. 2005), and remote sensing of cyanobacteria in natural waters (Simis et al. 2005). A large number of patents on fluorescence-based applications of phycobiliproteins reflect their growing demands in industry (Sekar and Chandramohan 2008). The price of phycobiliproteins or its components like phycocyanin may go up to thousands of US $ per milligram depending on their purity and intended use (Sekar and Chandramohan 2008).

A number of investigations have also shown the health-promoting properties and broad range of pharmaceutical applications of phycobiliproteins. The pharmacological properties like antioxidant, anti-inflammatory, and neuroprotective and hepatoprotective activity have been attributed to phycocyanin (Benedetti et al. 2004; Cherng et al. 2007). Phycocyanin has also been reported to reduce the levels of tumor necrosis factor (TNF-α) in the blood serum of mice-treated with endotoxin. Phycocyanin from *Aphanizomenon flos-aquae* has been described as a strong antioxidant (Bhat and Madyastha 2000; Romay et al. 2003), and its protective nature against oxidative damage has also been demonstrated in vitro (Benedetti et al. 2004).

As allophycocyanin (APC) is greater than 100 times more sensitive than conventional organic fluorophores, it is routinely used for flow cytometry, live cell staining, and multicolor immunofluorescent staining. Allophycocyanin (Molecular Probes®) from Invitrogen, PhycoPro™ PB25 Cross-Linked APC and Prozyme xl APC from Prozyme, Lightning-Link® Allophycocyanin (APC) from Innova Biosciences Ltd., Streptavidin cross-linked allophycocyanin conjugated from AnaSpec, etc., are some of the commercially available allophycocyanin-based products.

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