CHROMOPHORE-PROTEIN INTERACTION AND ENERGY TRANSFER
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LITERATURE: AN OVERVIEW

Bryant et al (1976), perhaps for the first time described the presence of PEC in *Anabaena variabilis* and *Anabaena* sp. 6411; a filamentous cyanobacterial species. Extensive work on this protein remained hampered till recently due to its limited occurrence in cyanobacteria (Bryant, 1982) and its being available generally in relatively low amounts. PEC carries one phycobiliviolinoid group on the \( \alpha \)-subunit and two phycocyanobilin chromophores on the \( \beta \)-subunit (Bishop et al, 1987). This unusual phycoviolobilin chromophore on the \( \alpha \)-subunit is responsible for the photochromic responses of PEC (Siebzehnrubl, 1989; MaruthiSai et al, 1992) as well as for the unique spectral behaviour. This whole phenomena can be envisaged within the broad spectrum of chromophore-chromophore and chromophore-protein interactions.

The photoreversible (photochromic) pigments such as phytochromes are the photoreceptors of plant photomorphogenesis. Many such photoreceptors have been reported to occur in various cyanobacterial species (Bjorn and Bjorn, 1976; Bjorn, 1978, 1980; Bogorad, 1975; Scheer, 1987; Tande de Marsac, 1983). These photochromic pigments are similar to the phytochromes of higher plants but these have absorption spectra with absorption maxima at lower wavelengths and hence are termed as phycochromes. Bjorn and Bjorn (1976) and later Bjorn (1978) reported spectral characteristics of four distinct photoreversibly photochromic pigments, the phycochromes a, b, c and d from various cyanobacterial species. Phycochrome exists in two forms under the influence of light quality. Under red light phycochrome ‘a’ has absorption maximum at 590 nm whereas phycochrome ‘a’ under green light show absorption maximum at 630 nm. Similarly phycochrome ‘b’ also exhibits two forms; one with absorption maximum at 510 nm and the other with its maximum at 570 nm in yellow
green light and blue-green light, respectively. Phytochrome 'c' appears to be less sensitive than phytochromes a and b. This contrast becomes more obvious under green/red light conditions; phytochrome 'c' formed under green light, absorbed maximally at 650 nm but the ones formed under red light showed a very weak absorption in the green region. The experimental evidence (Bjorn and Bjorn, 1976) on photochemistry of fractions obtained from several cyanobacterial species suggested that such photochromic changes correspond to the transformation of S-type chromophores into F-type chromophores in green light and F-type chromophores to S-type chromophores in red light. The photomorphogenetic responses of cyanobacteria are governed by a green/red antagonism marked by photoreversibility and resembles the classical phytochrome responses in higher plants with their red/far red antagonism (Bjorn, 1979). The photosynthetic antenna pigments of cyanobacteria, red algae and cryptophyte algae as well as the photomorphogenetic receptor pigments, phytochrome and phycochromes, carry open chain tetra-pyrrolic chromophores very similar in molecular structure (Grombein et al, 1975; Lagarias and Rapoport, 1980). Spectroscopic properties of these pigments provide an evidence regarding their covalent to their non-covalent interactions with the apoproteins which are very similar (Rudiger and Scheer, 1983). In vivo and isolated state properties of these two pigment classes are very similar. Some phycobiliproteins like PC and APC are chemically and photochemically inert. They are highly fluorescent in the isolated state but efficiently transfer excitation energy to the reaction centres in the native environment (Scheer, 1982). Contrary to this, phytochromes are photochemically very reactive and exhibit little fluorescence. Thummler and Rudiger (1983) studied some of the properties of the ZZZ and ZZ£ configurations of the chromophores of phytochrome and C-phycoecyanin. The designations Z and E refer to the configuration around the double bond connecting the individual pyrrole groups in the chromophore. Rudiger et al (1983), while studying the chromophore structure of the physiologically active Pfr form of phytochrome, discussed the possible relevance of these geometric isomers to phytochrome activity. They have also demonstrated phycocyanobilin chromophore being present in the ZZZ configuration. It was also possible to convert it to a ZZ£ isomer. Earlier, the studies of Scheibe (1972), on the extracts from the cyanobacterium Tolypothrix tenuis
which shows photoreversible behaviour and the ability to undergo complementary chromatic adaptation suggested that the photoconvertible pigment had two forms which were interconverted depending on whether red or green light was used for irradiation.

Ohad et al (1979, 1980) isolated a photoreversible pigment from the cyanobacterium N. muscorum and F. diplosiphon. Irradiation of isoelectric focusing fraction containing mixture of pigments with red light showed a small loss in 620 nm absorbance and a hyperchromicity at 560 nm. However, the green light irradiation of this mixture which was preirradiated with red light produced the reversal of the spectroscopic changes. These active fractions contained C-PE, C-PC and APC. These photoreversible fractions when passed through a protein-A Sepharose column containing antibodies specific to C-PC or C-PE, retained their activity but fractions passed through columns equipped with antiallophycocyanin antibodies became completely inactive. This experiment of Ohad et al suggested that APC is a photoresponsive pigment. Later, Ohad (1980) pointed out that the small absorbance changes may be possibly due to a minor component or a special state of allophycocyanin that served as the active element. Ohki and Fujita (1979a) irradiated both the extract and the whole cells from the cyanobacterium T. tenuis but did not observe any red/green photoreversible absorbance changes. Treatment of these extracts with 0.4 M guanidine hydrochloride could induce this type of reversibility. The high concentration of guanidine hydrochloride acted as an effective protein denaturant. Later, Ohki and Fujita (1979b) examined purified C-PC and APC in the absence of guanidine hydrochloride. These biliproteins lacked photoreversible behaviour, but as with the crude extracts, the biliproteins responded to 0.4 M guanidine hydrochloride. de Kok et al (1981, 1982) further reported that induction of photochemistry in C-PC and APC from Anacystis nidulans was also possible by using solvents like 0.75% ethylene glycol. It should be noted, that the properties of chromophores in the native state are drastically different from those of the free chromophores or denatured biliproteins, which are photolabile and chemically reactive, and have very different conformations. Schmidt et al (1988) while studying the ZZZ ↔ ZZE photoconversion of PC chromophores from Mastigocladus laminosus demonstrated that native PC is unreactive except for a slow, irreversible bleaching.
whereas photoreversible photochromic properties can be induced upon partial uncoupling of the chromophores by perturbation of the protein structure. They concluded that these reactions involve most likely $Z \leftrightarrow E$ interconversions of the chromophores at the 15, 16 double-bond between rings C and D, and also this reactivity is reminiscent of the primary photochemistry of phytochrome.

The photoreversible photochemistry of phycoerythrocyanin is reminiscent of the plant photoreceptor, phytochrome but with an orange/green rather than red/far red action spectrum (Bjorn, 1979; Kufer and Bjorn, 1989; Siebzehnruble et al, 1989; MaruthiSai et al, 1992). The isolated $\alpha$-subunit of PEC obtained from *Mastigocladus laminosus* and *Tolypothrii distorta Kutzing* var. symplocoides Hansgirg, strain IUCC424 (now UTEX424) showed the photoreversible photochemistry characterizing phycochrome b, a photoreversibly photochromic pigment confined thus far only to the extracts of phycoerythrocyanin-containing organisms (Kufer and Bjorn, 1989). Light induced absorbance changes in the $\beta$-subunit and phycoerythrocyanin of the same organisms have also been studied. Siebzehnrubl (1989) described in detail the photochemistry of PEC from *Mastigocladus laminosus* in their various aggregation states and observed a decrease in the photochemistry of larger aggregates. Siebzehnruble (personal communication) made studies on PEC from two cyanobacteria, e. g. *M. laminosus* and *Chroococcidipsis* sp. and have characterized the photochemistry of the pigment in different states of aggregation, and mutual interdependence of its phototransformation and aggregation. Very recently MaruthiSai et al (1992) characterized the phycoviolobilin chromophores of PEC obtained from *Westiellopsis prolifica* and *Nostoc rivulare* in both states. They also showed that reversible photochemistry is a useful analytical tool to detect PEC in cyanobacterial extracts.

The chromophore structure in biliproteins play an important role at various levels of chromophore-protein and chromophore-chromophore interactions. These chromophores (also called bilins) are linear tetrapyrroles (Lemberg, 1928, 1930) Lemberg and Bader (1933) showed that R-phycocerythrin contains a tetrapyrrolic prosthetic group closely related to the meso-bile pigments. They considered that their pigment was identical with a dehydrogenation product of mesobilirubinogen which
they named mesobilirhodin. The structure of the linear tetrapyrroles and the nature of the linkage to the protein has been a subject of investigation for many years. Till recently four isomeric tetrapyrroles have been distinguished by their absorption spectra in acidic aqueous solution: phycocyanobilin, 660 nm; a phycobiliviolinoid pigment, 590 nm; phycoerythrobilin, 555 nm; and phycourobilin, 495 nm (O'Carra and O'hEocha, 1976; Glazer, 1981; Scheer, 1982).

Structural studies on C-phycocyanin established that each of the three bilin chromophores was attached to the protein through a single covalent bond, a thioether linkage to a cysteinyl residues through the ethylidene group of ring A (Williams and Glazer, 1978; Lagarias et al, 1979). The bilins linked through both rings A and D to cysteinyl residues 10-residues apart in the linear amino acid sequence have also been reported.

The presence of a thioether linkage has been confirmed by NMR analysis of a bilin peptide derived by CNBr cleavage of C-phycocyanin (Lagarias et al, 1979). Crystallographic analyses of C-phycocyanin (Schirmer et al, 1985, 1986, 1987) and NOSEY studies of bilin peptides (Bishop et al, 1987) have further resolved the question concerning stereochemistry and linkage to protein. Arciero et al (1988c) studied the in vitro addition of bilins to apophycocyanin in order to examine the ability of the apoprotein to discriminate between different bilins. They established that apophycocyanin bound and reacted covalently with not only phycocyanobilin but also with biliverdin and phycoerythrobilin at the same sites as found earlier for phycocyanobilin (Arciero et al, 1988 b, a).

The dependence of various interactions of phycobiliproteins on the geometry of the chromophores is important so far as the quaternary structures of the phycobiliproteins are concerned. Various types of chemical modifications of chromophores have been employed so as to expand the understanding of chromophore-protein interactions in different phycobiliproteins. Kufer and Scheer (1979, 1982) chemically modified the chromophores of C-phycocyanin and C-phycoerythrin by reduction using sodium dithionite and sodium borohydride. Recently Fischer and
Scheer (1992) demonstrated the effect of phycocyanobilin chromophore modifications on C-phycoerythrin heterohexamers. They modified bilin chromophores of the \( \alpha \)-or \( \beta \)-subunits of C-PC from *Mastigocladus laminosus* and subsequently recombined with the respective complementary unmodified chromophores. They brought about modifications either by photobleaching (350 nm) or by reduction of the verdin-to-rubin-type chromophores and observed that recombination did lead to heterodimers \((\alpha\beta)_1\), but the heterohexameric aggregation state could not be achieved with the modified chromophores. They have also demonstrated that autooxidation of the reduced \( \alpha_{\text{a}} \) chromophore in such hybrid induced reaggregation to heterohexamers. Chemical re-oxidation of the reduced chromophores however did not produce reaggregation. Thus, they showed that the intact chromophore structure is an important factor determining the quaternary structure of biliproteins and chromophore modifications also have a profound influence on chromophore-protein interactions and chromoprotein aggregation.

The absorption properties or the colour of a given PBP is not determined solely by the chemical nature of its chromophores. In fact, the conformation and environment imposed on the chromophore by the native protein do have strong influence over the spectroscopic properties of each chromophore. An important contribution to this also comes from the inter-\( \alpha\beta \) interaction and this could be the reason so as to why monomeric \((\alpha\beta)\) allophycocyanin has an absorption maximum at 615 nm whereas absorption maximum of trimeric APC \((\alpha\beta)_3\) lies at 650 nm. According to Scheer (1982), the chromophores in the native biliproteins are probably rigidly fixed in extended and twisted conformation near the surface of the protein. He also suggested energetically unfavourable conformations must be stabilized by noncovalent interactions with the polypeptide chains. Later a detailed account on this rather general picture on chromophore conformation was given by x-ray analysis of phycocyanin (Schirmer et al., 1985, 1986, 1987). The phycocyanobilin chromophore located on the \( \alpha \)-subunit of PEC have shown a mutual interdependence of phototransformation and aggregation (Siebzehrubl et al., 1989; MaruthiSai et al., 1992) and the molecular mechanism involved is suggested to be photoisomerization of the double bond between tetrapyrole rings C and D of the chromophore. Crystallographic details of Schirmer states that the location of an
aspartate residue next to the bile pigment (i.e. in all the three types of PCB chromophores) in the different subunits of PC in different cyanobacteria and red algae is conserved. And the same has been found to be true in the case of phycoerythrocyanin (PEC) from *Mastigocladus laminosus* (Duerring et al., 1990). While investigating the chromophore-protein interactions using a force field, Schamgl and Schneider (1991) showed that the interaction with the protein such as the formation of salt bridge, and the presence of different tautomeric forms of amino acids with carboxylate or amino and imino functions are essential for the tuning of the visible absorption band to a region where effective collection of light in the antenna complex may occur. Grombein *et al.* (1975) have confirmed experimentally the existence of a protonated chromophore in PC by observing a pKa value approaching '5'. Using quantum-mechanical model calculations Schamgl (1986) has given a general description of the effect of protonation on the electronic structure of the chromophore and suggested that the protonated chromophores in both ground and excited states may have similar type of chromophore-protein interactions and also to the extent that the molecular orbitals and excitation energies of the protonated chromophores are more sensitive to interaction with charges of different sign.

Chromophore environment in the native phycobiliprotein play an important role in the capture and transfer of excitation energy from one phycobiliprotein to another. Isolated phycobiliproteins and phycobilisomes are highly fluorescent, and this is essential for their biological function. Any of the factor influencing fluorescence are interesting for the understanding of these interactions, and of energy transfer both within the phycobiliproteins and from the latter to the chlorophylls in the photosynthetic membrane. Very often fluorescence and absorption spectroscopy were performed on purified biliproteins, phycobilisomes and intact whole cells at very low temperatures, commonly at -196°C (the temperature of liquid nitrogen). This procedure led to significantly narrower the spectral bands and a better resolution of overlapping spectra (Harnischfeger and Codd, 1978; Schreiber *et al.*, 1979; Canaani and Gantt, 1980; Gray and Gantt, 1975; Frackowiak and Grabowski, 1971; Peterson *et al.*, 1981; Krasnovsky and Kovaler, 1981). Results of low temperature fluorescence of Mimuro and Fujita (1980) indicated that content of various APC forms varies with the species or strain of cyanobacteria.
and the energy transfer at the phycobilin level is directly from APC to PS II chlorophyll ‘a’ when the special form of APC is present in low amount.

Gantt et al (1979) studied the low temperature fluorescence of intact PBSome from blue-green algae and red-algae and obtained their fluorescence emission peak at 678-685 nm.

In PBSomes the energy is sequentially conveyed from short wavelength absorber to those at longer wavelengths and finally it reaches the Chl ‘a’ in the reaction centre. This type of energy migration is assumed to occur by resonance transfer. For an effective resonance transfer the fluorescence emission of donors must overlap with the absorption spectrum of the acceptor (Forster, 1948, 1967). Furthermore, for maximal transfer the donor and receptor molecules should be in close proximity. PBSomes, a supramolecular and ordered complex of phycobiliproteins, fulfill such requirement and thus exhibit efficient energy transfer to PS II reaction centres. Energy transfer in phycobiliproteins is still only partly understood. Sauer et al (1987) modelled the excitation energy transfer in C-phycocyanin using the Forster inductive resonance mechanism. They also estimated the spectral overlap integrals between various chromophores of C-phycocyanin of Mastigocladus laminosus using the absorption and fluorescence spectra of C-phycocyanin and its separated subunits. Later Sauer and Scheer (1988) calculated the excitation transfer rates among the chromophores of C-phycocyanin using the previous method but with the help of new chromophores (Schirmer et al, 1987).

This chapter mainly deals with the chromophore-protein interaction with respect to the chromophore conformation and energy transfer.

RESULTS

The constituent PBPs of PBSomes from Nostoc rivulare have been characterized as described in the previous chapters. In this process we have also investigated the role of (PBPs) as excitation energy acceptor and donor in the energy transfer chain. This chapter is mainly devoted to certain aspects of chromophore-chromophore and
Fig. 7.0 PEC (reddish-blue band) and PBSomes visualised together on sucrose density step gradient. Sucrose gradients were prepared as described in Materials and Methods.
chromophore-protein interaction. These interactions will be investigated with respect to their role in energy transfer mechanism.

After we have established the presence of PEC in *Nostoc rivulare*, our aim was to confirm the identity of the reddish blue band that was observed in the region of 0.5M to 0.25M on the sucrose density step gradients during isolation PBSomes. Careful elution and steady-state spectroscopic analysis of this band indicated that it was enriched with PEC (Fig. 7.1). Though this fraction was present in 1M K-phosphate buffer (pH 7.0), it closely resembled with trimeric PEC (Bryant *et al.*, 1976; Bryant, 1982; Fuglistaller *et al.*, 1981) in both its absorption and fluorescence properties. Dialysis of this PEC enriched fraction (1M) against 5mM K-phosphate buffer (pH 7.0) resulted in a species that absorbed maximally at 604nm along with a blue shoulder (Fig. 7.2) and resembled a monomeric PEC (MaruthiSai *et al.*, 1990). We could observe the appearance of PEC enriched band in the step gradient only when the hour of ultracentrifugation was prolonged.

The presence of PXB chromophores in PEC makes it readily photoreactive in its native state (Kuffer and Bjorn, 1989). Bjorn (1979) traced a relationship between phycochrome b, so far identified only in PEC containing cyanobacteria, and the α-subunit of PEC. The results obtained by MaruthiSai *et al.* (1992) indicated that reversible photochemistry can be sensitive analytical tool to detect PEC in cyanobacteria. We, therefore, have further characterized the PEC present in the reddish-blue band as well as in the PBSome by exploiting the photochemical responses of this biliprotein. In order to obtain photoreversible photochromic response, we made certain modifications depending on the limitations in the instrumentation. To this effect, we have used orange (581 nm)/green (520 nm) light instead of either orange (600 nm)/green (500 nm) [MaruthiSai *et al.*, 1992] or orange (570 nm)/green (510 nm) [Kufer and Bjorn, 1989]. Intact PBSomes (*Nostoc rivulare*) did not show any photochemical response. Figure 7.3 shows an absorption difference spectrum of dissociated PBSome with its positive and negative extremes at 502 and 565 nm respectively. A comparative absorption difference spectra of dissociated PBSomes from *Nostoc rivulare* and *W. prolifica* upon induction of photochemistry by orange (581 nm) light, is shown in Fig. 7.4. This also
Fig. 7.1. Absorption spectra of PEC enriched fraction (1M).
Fig. 7.2. Absorption spectra of PEC enriched fraction after dialysis (overnight) against 5mM K-P buffer, pH 7.0.
exhibits a complete reversal of photochemistry by green (520 nm) light. Fig. 7.5-6 deal with the induction and reversal of photochemistry of PEC enriched fraction and PEC trimer upon orange (581 nm)/ green (520 nm) irradiations respectively. One major problem we came across while recording the absorption difference spectra was the signal to noise ratio. In order to improve upon it, the data points at each wavelength across the entire scanning range, were collected five times and the average of it at each wavelength were plotted as the absorption difference spectrum. Table: 7.1 illustrates the amount of photochemical response obtained for various PEC containing samples.

The occurrence of photochemical responses in PEC has been attributed to phototransformations that take place at the level of PXB chromophore (Kufer and Bjorn, 1989). Similar photochemical responses were not observed in other PBPs (e.g. PC and APC) in their native states. To obtain further knowledge about the various interactions occurring during excitation energy transfer, we have studied the spectroscopic properties of tetrapyroles (chromophores) both in free as well as in the sterically constrained states at the various levels of aggregations. Fig. 7.7 deals with the absorption spectra of a free bilin (represented by biliverdin dihydrochloride), bilins in denatured PEC and bilins in PEC trimer. Likewise, Fig. 7.8 deals with the absorption spectra of trimeric and denatured PC and APC forms indicating the status of chromophores in them. To deduce the probable conformation of chromophores (bilins) at various levels of chromophore-protein interactions, we have plotted visible to near UV absorbance ratios as a function of aggregation state of PBPs. To this effect, Fig. 7.9 (a,b,c) deals with the plots of visible to near visible absorbance ratios vs aggregation for PEC, PC and APC. The proper understanding of the mechanism of energy transfer would not be complete without developing the knowledge about the emissive properties of PBSomess and its constituent PBPs at low temperature. Low temperature fluorescence led to a better resolution of overlapping spectra. Figure 7.10 (a,b,c,d) and table 7.2 deal with the low temperature (-196°C) fluorescence of PBSomess, PEC, PC and APC. On the basis of the information available to us from previous chapter (steady-state spectroscopy), we have simulated the spectral overlaps between donors and acceptors.
Fig. 7.3. Absorption difference spectra of dissociated PBSome (Nr).

Trace: Absorption difference spectra of 5 min 581 nm illumination minus 8-10 min. saturation by 520 nm pre-irradiation.
in the energy transfer chain. In this regard Fig. 7.11 shows the spectral overlaps of absorption and emission spectra for PEC, PC and APC. Table 7.3 represents the calculated overlap integrals between various donors and acceptors.

On the basis of all information derived by us from our present studies, we propose, henceforth, a more comprehensive scheme of energy transfer in PEC containing organisms (Fig. 7.12).

**DISCUSSION**

**Photochemistry:**

Phycoerythrocyanin shows photoreversible photochemistry, which has been attributed to phycoviolobilin chromophore in its α-subunit (Kufer and Bjorn, 1989; Siebzehnrubl, 1989). The suggestion that the induction of photochemistry of PEC can be used as a sensitive assay for its presence in cyanobacteria (MaruthiSai et al., 1992) helped us in confirming our earlier submission about the presence of PEC in *Nostoc rivulare* as one of the constituents of PBSome. The reddish-blue band that appeared in 0.5M to 0.25M region of the step-gradient during PBSome isolation procedure, showed photochemistry upon orange (581 nm)/ green (520 nm) irradiation. Further, the steady-state absorption and emission properties matched with those of trimeric PECs (100mM K-phosphate buffer) [Bryant et al., 1976; Bryant, 1982; Fuglistaller et al., 1981] obtained after DEAE-cellulose and hydroxyapatite column chromatography. The reddish-blue band contained 1M K-phosphate buffer (pH 7.0). This fraction/band upon overnight dialysis against 5 mM K-phosphate buffer (pH 7.0) at 4°C, exhibited absorption and emission properties similar to monomers obtained from 100 mM trimeric PEC. From the results of steady-state absorption, fluorescence and photochemistry we conclude that the reddish-blue band contained PEC. However, a small amount of PC may be present as contaminant. By careful preparation this amount may be reduced to too low an amount to be significant. However, for the sake of convenience we tentatively describe the reddish blue band as PEC enriched fraction or band. Earlier we suspected this PEC enriched band as either being an artifact or present as an extracellular entity. By cautious checking of the appearance of PEC
Fig. 7.4. A comparative absorption difference spectra of dissociated PBSomes

Trace 1: Dissociated PBSome (Nr): Absorption difference spectra of 5 min 581nm illumination minus 8-10 min saturation by 520nm pre-irradiation.

Trace 2: Dissociated PBSome (Wp): Absorption difference spectra of 5 min 581nm illumination minus 8-10 min saturation by 520nm pre-irradiation.

Trace 3: Dissociated PBSome (Nr): Absorption difference spectra of 5 min 581nm illumination followed by 8-10 min 520nm pre-irradiation minus 8-10 min 520nm pre-irradiation.
enriched band at various time intervals of centrifugation and after carefully monitoring the mechanical agitation steps during PBSome isolation procedures, we conclude that the origin of PEC as a reddish-blue band in the 0.5M to 0.25M region of step gradient infact lies in the intense blue band of PBSomes which is located in the 1.0 M region on the step gradient and not from any other source as suspected earlier. Also, perhaps the prolonged hours of ultracentrifugation causes the release of PEC from the tips of the PBSomes. We observe at the end of this entire exercise emergence of a new isolation procedure of crude PEC and PBSomes, simultaneously.

The photochromic characteristics of α- subunit of PEC both in free and in various aggregates resembles with that of the phycochrome b activities detected in certain cyanobacteria (Kufer and Bjorn; 1989). In analogy to phycochrome, the probable molecular mechanism involved in photochemistry is a $Z \leftrightarrow E$ photoisomerization of the double bond between the ring C and D (15) of the tetrapyrrole (PXB). Siebzehnrubl et al (1989) have established a mutual interdependence between phototransformation and aggregation in case of PEC. PEC is very prone to dissociation compared to other PBPs (Bryant, 1982).

Our study on photochemistry is mainly concerned with the conformational changes occurring at the level of PXB chromophores due to the changing environment of PEC. We could not detect a photochemical response for intact PBSomes upon orange (581 nm)/green (520 nm) irradiation whereas the dissociated PBSomes, PEC enriched band and trimeric PEC, all of them showed the characteristic positive and negative extrema, albeit with a little variations (table 7.1). A comparative absorption difference spectrum of dissociated PBSomes from Westiellopsis prolifica and Nostoc rivulare did not show any significant change in their respective level of photochemistry upon orange (581 nm)/green (520 nm) irradiation. Trimeric PEC showed a photochemistry about 22% which is ∼4% more than what has been observed for trimeric PEC from Mastigocladus laminosus (18%, Siebzehnruble et al, 1989) and Westiellopsis prolifica (17%; MaruthiSai et al, 1992). Such variations have also been observed for monomeric PEC. In case of Mastigocladus laminosus the photochemistry observed was 36% (Siebzehnrubl et al, 1989) whereas the same was found to be 41% (as high as 5%)
Fig. 7.A Structures of phycoviolobilin chromophores in their 102, 15E-configuration (right). Schematic, native chromophores have extended geometries.
Fig. 7.5: Absorption difference spectra of PEC enriched fraction (1M).

Trace 1. Absorption difference spectra of 5 min 581nm illumination minus 8-10 min saturation by 520nm pre-irradiation.

Trace 2. Absorption difference spectra of 5 min 581nm illumination followed by 8-10 min 520nm pre-irradiation minus 8-10 min 520nm pre-irradiation.
Fig. 7.6: Absorption difference spectra of PEC Trimer.

Trace 1. Absorption difference spectra of 5 min 581nm illumination minus 8-10 min saturation by 520nm pre-irradiation.

Trace 2. Absorption difference spectra of 5 min 581nm illumination followed by 8-10 min 520nm pre-irradiation minus 8-10 min 520nm pre-irradiation.

Our observations on photochemistry of PEC in different environment is generally in agreement with those reported for Mastigocladus laminosus (Siebzehnrubl et al, 1989) and Westiellopsis prolifica (MaruthiSai, 1990). Although we did not witness any detectable PEC photochemistry in intact PBSome, a negligible photochemistry of 0.31-0.36% has been reported for intact PBSomes from Mastigocladus laminosus by Siebzehnrubl et al (1989). According to them, the possible reasons for reduced photochemistry in higher aggregates could be

a) the decreased mobility of the α-PXB chromophores of PEC due to the compact packing of all the chromophores which inhibit the rotation of ring necessary of isomerisation,

b) due to the competition of energy transfer to lower energy phycocyanobilin (PCB) chromophore with photochemistry. The results regarding photochemistry of intact and dissociated PBSomes from Nostoc rivulare (Table 7.1) supports both the mechanisms suggested by Siebzehnrubl et al (1989).

The energy transfer in intact PBSome is highly efficient and occurs at picosecond time scale wherein the excitation energy is passed onto the terminal emitters. It may be due to this ultrafast energy transfer that we do not witness any detectable photochemistry.

On the other hand we know that the dissociation of PBSomes causes reduction in the non-covalent interaction between the chromophore and apoproteins which inturn enhances mobility of α-PXB chromophores. This PXB mobility increases the possibility of Z ↔ E isomerization of the double bond between ring C and D. Hence, we notice an increase in photochemistry of dissociated PBSomes. This view of ours is in agreement with other workers (MaruthiSai, 1991). Furthermore, the percentage photochemistry of 20.6% for the reddish-blue band (1M K-phosphate buffer, pH 7.0) nearly agrees with the percentage photochemistry of ~22% obtained for trimeric PEC. This similarity further confirms that the reddish-blue band is predominant with PEC.
Table [7.1]:

Amplitudes and Absorbtion difference extrema of samples containing PEC.

| Sample                              | Buffer | A at $\lambda_{max}$ | $|\Delta \Delta A|$ | $\Delta \Delta A \times 100$ | Difference extrema
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>PBSome intact (Nr)</td>
<td>1M</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBSome dissociated (Wp)</td>
<td>5mM</td>
<td>0.2</td>
<td>6.9x10^{-3}</td>
<td>3.45</td>
<td>502 565</td>
</tr>
<tr>
<td>PBSome dissociated (Nr)</td>
<td>5mM</td>
<td>0.2</td>
<td>6.1x10^{-3}</td>
<td>3.05</td>
<td>502 565</td>
</tr>
<tr>
<td>PEC enriched fraction</td>
<td>1M</td>
<td>0.24</td>
<td>5.96x10^{-3}</td>
<td>22.98</td>
<td>502 567</td>
</tr>
<tr>
<td>PEC trimer</td>
<td>100mM</td>
<td>0.24</td>
<td>1.32x10^{-3}</td>
<td>20.66</td>
<td>502 567</td>
</tr>
</tbody>
</table>
Chromophore conformation & Energy Transfer:

The properties of individual chromophores in the native biliprotein environment play an important role in harvesting and transfer of light energy. These chromophores differ considerably in their spectroscopic behaviour. The two bilin chromophores of our interest in the present investigation are phycocyanobilins (PCB) and phycoviolaobilin (PXB). All the PCBs are chemically the same whereas PXB chromophores differ only in terms of the length of the conjugated system which remain shorter than the PCBs but longer than phycoerythrobilins (Bishop et al., 1987). However the chemical structure of PXB is still not well established. These variations influence the directional flow of excitation energy in native biliprotein (Mimuro et al., 1986; Sauer and Scheer, 1988). Each chromophore has a characteristic absorption maximum in the near UV region in addition to the one in the visible region. This fact is also reflected in the absorption spectrum of native biliproteins. The PCBs show a characteristic near UV absorption maximum at ~345 nm whereas PXBs have characteristic near UV absorption maximum at ~332 nm. PEC, which contains both PXB and PCB chromophores, upon denaturation shows a characteristic near UV absorption maximum at ~332 nm along with a red shoulder at ~345 nm (Fig. 7.7b). Here, 332 nm peak is characteristic of PXB whereas 345 nm peak is characteristic of PCB chromophore. We have used these special characteristics of PXB and PCB chromophores to obtain information about their relative chromophore conformation in various phycobiliprotein aggregates and/or in different environments with respect to the chromophore-protein interaction (Schnieder et al., 1988; Scharngl and Schneider, 1989).

X-ray diffraction studies (Schirmer et al., 1985, 1986, 1987) and later crystallographic studies by Duerring et al., 1990) have shown that the bile pigment chromophores such as PCBs in PC and PXB in PEC, assume fairly stretched conformation. Upon denaturation of biliproteins, the uncoupled chromophores as well as free bile pigments of similar structures represent flexible chromophores which have predominantly a cyclo-helical conformation in solution (Scheer, 1982; Braslavsky et al., 1983). Thus, the absorption spectra of biliverdin dihydrochloride and of chromophores that are released upon denaturation of phycobiliproteins (PEC, PC and APC), as illustrated
Fig. 7.7: Absorption spectra of
a) biliverdin dihydrochloride
b) denatured PEC (0.1% SDS + 1 min heating)
c) PEC Trimer

a & b represent chromophores in cyclic state whereas c represent the chromophores in extended or stretched conformation.
Fig. 7.8: Absorption spectra of
a) PC and APC Trimer
b) denatured PC (0.1% SDS + 1 min heating)
c) denatured APC (0.1% SDS + 1 min heating)
a represents chromophores in extended or stretched conformation where
b & c represent the chromophore in cyclic conformation.
in Fig. 7.7b and 7.8b & c, represent cyclic-helical conformation. The absorption spectra of native PEC, PC and APC (Fig. 7.7c & 7.8a) indicate the PXB and PCB chromophores being in stretched or extended conformation.

In extended or stretched geometry the transition moment is increased and therefore, the absorption probability in the visible region is enhanced with respect to the UV band (Scheer, 1982). The cyclo-helical conformers are characterized by a strong near UV absorption band and weaker or moderate absorption in the visible region (Bradlmeir et al, 1981). Synthesized tetrapyrroles that are constrained by covalent bonds to be in an extended conformation show their visible absorption bands being strong absorbers whereas the near UV absorption bands absorbing very weakly (Bois-Choussy and Barbier, 1978). Further, we have explored the proposal made by Scheer and Kufer (1977) regarding the conformation of PCB chromophores of C-PC, in our present investigation of deducing relative conformation of PXB and PCB in various phycobiliprotein (PEC, PC and APC) aggregates and/or in different environments. Thus, the ratio of visible to near UV absorbance becomes an indicator of chromophore conformation. To this effect, we have plotted the ratios of visible to near UV absorbance as a function of chromophore environment (both in free state as well as in various levels of their aggregation). Fig. 7.7a,b & c describe the changing conformation of chromophore from cyclic to linear state in different apoprotein environments.

The conformation of chromophores (PXB and PCB) in the phycobilisomes are achieved in discrete steps (Fig. 7.9a,b & c). For PEC, the visible to near visible absorbance ratios of biliverdin dihydrochloride, which represents free chromophore, is the lowest ($A_{690}/A_{321} = 0.436$; $A_{690}/A_{375} = 0.414$). This is then followed by the ratio calculated for PXB and PCB in the denatured protein, where the tetrapyrroles are relatively free of non-covalent interactions with the apoprotein and are thus able to achieve their own stable status. When denatured PEC was compared to monomers, we observed a large increase in the visible to near visible absorbance ratio(s) for both PXB (1.93) and PCB (2.85) which indicates the bilin chromophores are significantly more extended than those in the denatured PEC. Further PCBs appear to be relatively more extended than the PXB. The equilibrium mixture of monomers and trimers of
Fig. 7.9a  Tetrapyrroles (PXB & PCB) of PEC: Relative tetrapyrrole (Chromophore) conformation as a function of changing polypeptide (apoprotein) environment.

The subunit value is for subunits in the denatured condition of PEC. BVD, Biliverdin dihydrochloride; M, Monomer; MT, Monomer-Trimer equilibrium mixture; T, Trimer; PBSome, Phycobilisomes.
PEC showed a significant increase in the visible to near UV absorbance ratio for PXB (2.85) but we did not observe any significant change for PCB in similar conditions. Examination of trimers revealed that PXB and PCB were in further extended state. Studies on intact PBSomes indicated that the conformational state of PXB in the trimers determined the final conformation of PXB chromophores of the PEC whereas the conformation of PCB chromophores of the PEC assumed more stretched conformation than those of the trimeric PEC.

PC and APC carry only PCB chromophores. In APC, PCB of the monomers exhibit comparatively more extended conformation than the chromophores in denatured PBPs. Trimeric APC seems to have slightly stretched PCB chromophores than the monomers. Before assuming final conformation state in PBSomes, PCB chromophores of APC are further extended. The PCBs of C-PC also undergo similar conformational changes before assuming final conformation in PBSome. The conformational state achieved by PCB chromophores of C-PC at the hexameric level of aggregation is maintained as the final conformation of PCB chromophore of C-PC in PBSome.

The extended chromophore geometry is favoured by interaction of chromophore with the protein and the required amount of stabilizing energy comes from electrostatic interactions, particularly the formation of a salt bridge with a nearby aspartate residue (Schramagl and Schneider, 1991), which is conserved in all phycobiliproteins studied so far by x-ray crystallography (Schirmer et al, 1985, 1986, 1987; Duerring et al, 1990). Both free bile pigments and chromophores in denatured PBPs fluoresce very weakly (Braslavasky et al, 1983). This indicates that excitation energy is lost rapidly by non-radiative relaxation pathways such as vibrations or rotations about single bond (Kufer et al, 1983), photoisomerization (Siebzehnrubl et al, 1989), intersystem crossing and proton transfer (Falk and Neufingerl, 1979; Friedrich et al, 1981; Gerwert et al, 1990). These deexcitation pathways are minimized by the environment and rigid extended conformations of the chromophores in the native PBPs. Consequently the native PBPs are highly fluorescent (see chapter steady-state spectroscopy). We conclude that chromophore-protein interactions determine the conformation of chromophores and the non-covalent interactions with the polypeptides are necessary for the stabilization
Fig. 7.9b  Tetrapyrroles (PCB) of PC: Relative tetrapyrrole (Chromophore) conformation as a function of changing polypeptide (apoprotein) environment.

The subunit value is for subunits in the denatured condition of PC. BVD, Biliverdin dihydrochloride; M, Monomer; T, Trimer; H, Hexamer; PBSome, Phycobilisomes.
Fig. 7.9c  Tetrapyrroles (PCB) of APC: Relative tetapyrrole (Chromophore) conformation as a function of changing polypeptide (apoprotein) environment.

The subunit value is for subunits in the denatured condition of APC. BVD, Biliverdin dihydrochloride; M, Monomer; T, Trimer; PBSome, Phycobilisomes.
of energetically favourable conformation in native PBPs as well as in PBSomes. Thus, we observe that the molecular ecology of chromophores is very important for the capture and efficient transfer of excitation energy in the PBSomes. Our studies on Nostoc rivulare have revealed that PEC is the first pigment-protein in the energy transfer sequence. The excitation energy captured by PEC is transferred to PC, after leaving PC, energy migrated to APC. Whether or not does the further migration of excitation energy from APC to Chl ‘a’ need the mediation of a long wavelength emitter, such as APC B? In our studies APC showed an emission maximum of 663 nm at room temperature whereas the intact PBSome preparations showed an emission maximum of 670 nm at room temperature and this indicates a possible mediation by long wavelength emitters during the migration of energy from APC to Chl ‘a’. Gantt et al (1977) suggested a contribution from APC B in the case of Porphyridium cruentum, only as a small fraction to the total emission intensity at 685 nm. Although we did not succeed in separating the long wavelength emitters probably due to its presence in extremely low amount so that it could not be detected even by absorption spectroscopy. However, low temperature studies at liquid nitrogen temperature (-196°C) showed a fluorescence emission maximum of ~682 nm for intact PBSomes (fig. 7.10a). Low temperature fluorescence studies on Anacystis nidulans and Cyanidium caldarium by Rijgersberg and Amesz (1980) suggested that the fluorescence increase upon cooling can be explained by a lowering of the efficiency of energy transfer between chlorophyll molecules. They also concluded that a small percentage of the emission at 685 nm can be ascribed to APC B, and that the energy transfer from APC to APC B in isolated PBSomes occurs with an efficiency of about 90% at low temperature. Furthermore, partially dissociated PBSome preparations of Nostoc rivulare at low temperature (-196°C) (fig. 7.10a) show three emission leakage points in the emission spectrum which indicated a prominent emission at 648 nm along with a blue (~620 nm) and a red (~670 nm) shoulders representing partial emission losses from PC, PEC and APC respectively. Mimuro and Fujita (1980) reported that in those species or strains of cyanobacteria where the amount of the special forms of APC is low, part of the energy transfer at the phycobilin level was directly from APC to PS II Chl ‘a’. The informations obtained from Nostoc rivulare strongly suggests the
Fig. 7.10. Low Temperature (-196°) Fluorescence emission spectra of PBSomes and phycobiliproteins from Nostoc rivulare.

a) PBSome Intact and partially dissociated with λex at 545nm.
b) PEC Trimer with λex at 545nm.
c) PC Trimer with λex at 580nm.
d) APC Trimer with λex at 600nm.

Each sample contained 30% Glycerol to the final concentration.
Table [7.2].

Low Temperature (-196°) of PBP's and PBSomes of *Nostoc rivulare*

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda_{ex}$ (nm)</th>
<th>$\varepsilon_{max}$ (nm)</th>
<th>Shoulders blue</th>
<th>Shoulders red</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEC</td>
<td>545</td>
<td>530</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC</td>
<td>545</td>
<td>650</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APC</td>
<td>580</td>
<td>650</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBSome (partially dissociated)</td>
<td>600</td>
<td>663</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBSome (intact)</td>
<td>545</td>
<td>648</td>
<td>620</td>
<td>670</td>
</tr>
</tbody>
</table>
possible involvement of long wavelength emitters such as APC B, APC I during energy migration from APC to chl 'a'. We, therefore propose the possible routes of energy migration from PEC to Chl 'a' in Nostoc rivulare as under:

PEC$\rightarrow$PC$\leftarrow$APC II$\rightarrow$APC B$\leftarrow$95 KD polypeptide$\rightarrow$Chl 'a'

Later, fluorescence emission spectra obtained at liquid nitrogen temperature for phycobiliproteins of Nostoc rivulare (Fig. 7.1Ob, c, d) indicated the spectral band being significantly narrower and also a better resolution of overlapping spectra was possible.

If the criterion of spectral overlap between the donors and acceptors as laid down by the Forster mechanism of energy transfer, is fulfilled, the route of energy migration as mentioned above may not be required in the concept of excitation energy transfer (Forster, 1948, 1967) In Fig. 7.11, the fluorescence emission spectrum of PEC overlaps absorption spectra of PC and APC to a considerable extent. Thus emission from PEC fulfills the criterion of spectral overlap with the absorption of APC and the calculated value of the overlap integral (J) between PEC and APC is $2.73 \times 10^{-10}$ cm$^6$/mol whereas the calculated value of J between PEC and PC is $6.95 \times 10^{-10}$ cm$^6$/mol (see Table 7.3). Grabowski and Gantt (1978) compared the emission of PE with that of PC and APC. They concluded that PE were spectrally more suited for direct transfer to an APC as to a PC acceptor only if both the PC and APC acceptors were at the same distance and orientation from the donor. In case of Nostoc rivulare, though the value of J between PEC and PC as well as between PEC and APC, similarly, suggests a direct transfer to APC only if the distance and orientation between PEC and APC, and PC and APC remained same, which is certainly not the case. Gantt et al (1976) proposed the structural design of the PBSome where PC was physically placed between PE and APC for the total specificity of PC as the intermediary. This proposal also seems to be true for PEC containing PBSomes from Nostoc rivulare. Therefore, the importance of stepwise transfer from PEC appears to be two-fold; first, it improves the extent of spectral overlap for the next transfer, and second, it provides, perhaps more critically, a unidirectional flow by setting up controlled intermediary steps which.
Fig. 7.11  Overlapping of absorption and emission spectra of constituent phycobiliproteins of Nr PBSomes. All the spectra were obtained from the chapter: steady state spectroscopy.
Table [7.3].
Overlap Integrals* (x10\(^{10}\), Cm\(^{4}\) mol\(^{-1}\)) calculated for constituent PBPs of PBSomes (*Nostoc rivulare*).

<table>
<thead>
<tr>
<th>Donor</th>
<th>PEC</th>
<th>PC</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEC</td>
<td>4.81</td>
<td>6.95</td>
<td>2.73</td>
</tr>
<tr>
<td>PC</td>
<td>1.08</td>
<td>2.94</td>
<td>3.39</td>
</tr>
<tr>
<td>APC</td>
<td>0.35</td>
<td>1.08</td>
<td>2.59</td>
</tr>
</tbody>
</table>

* Overlap Integral (J) calculated from expression:

\[
J = \int_{0}^{\infty} F_D(\lambda)E_A(\lambda)\lambda^4 \, d\lambda
\]
would check the backward migration of energy towards the periphery of the PBSome.

Table 7.3 lists the values of ‘J’ for various donor-acceptor pairs of PEC containing PBSome of *Nostoc rivulare*. The relatively low value of overlap integral between a C-PC donor and a PEC acceptor and similarly low J values between an APC and donor and a C-PC or PEC acceptor suggests that back transfer from a lower to higher energy biliprotein seems to be less probable than PEC transferring energy to another PEC where the ‘J’ value for self transfer is quite significant \((4.81 \times 10^{-10} \text{ cm}^6/\text{mol})\).

**Energy Transfer: A Scheme**

PBSomes are optimized for efficient energy transfer, which proceeds energetically downhill from the periphery to the core and ultimately feed the photosystem II reaction centres when they are attached to the photosynthetic membrane in vivo conditions. It has been found that the transfer of excitation energy takes place on a picosecond time scale. We, too, observed a rod to core transfer time in the range of 50-197 ps for intact PBSomes. Although, in our studies we could not achieve better resolution of \(S \rightarrow F\) transfer time for isolated individual PBPs, however, the fast decay components in such cases, clearly indicated the transfer is ultrafast, occurring at picosecond time scale. There is very little information available so far on the energy transfer process within PBSome core complex. Successful attempts have been made to develop a full understanding of the energy transfer processes in ‘simple’ PBSomes with homogenous rods, such as *Synechococcus 6301* (Glazer, 1983).

On the basis of the information obtained from our present investigation on complex PBSomes (PEC containing), we have tried to gain an insight into the mechanism of energy transfer in PBSomes with heterogeneous rods, i.e., rods containing PEC. To this effect, we propose a comprehensive scheme of energy transfer in PEC containing PBSomes of *Nostoc rivulare*. Thus, we started with a fairly simple model and later went on adding more complexity to it so as to finally arrive at a scheme that adequately explains the energy transfer processes in such systems. Fig. 7.12 illustrates the Scheme where each of the three rectangular compartments represents PEC, PC and APC, respectively. Each of the compartments is further divided into
Fig. 7.12. A Scheme of Energy Transfer in PBSome with heterogenous rods (containing PEC).
sub compartments indicative of S and F chromophores. The areas marked with dashed lines represent the emissions from various PBPs. In order to have satisfactory description of the energy transfer processes through such scheme following assumptions are made:

a. It is assumed that the rod to core transfer of energy is through identical APC chromophores present near the rod-core junction.

b. The rod to core energy transfer kinetics is assumed to be identical for each rods of the PBSome and this transfer takes place by Forster mechanism.

c. Downhill transfer of energy from PEC—> APC is possible because of the favourable spectral overlaps whereas back transfer from the core to the rod is excluded partly due to the small overlap integrals for such steps and partly because initial transfer to APC is followed by an additional fast transfer step to Terminal emitters, which both quickly depopulates the APC chromophores at the rod-core junction.

d. There is no reliable information on the existence of higher aggregates (more than trimer) for PEC and APC in isolated condition and therefore, we have calculated the overlap integrals in terms of trimers of acceptors and donors. In order to have uniformity in representation, we consider each of the compartments along with their associated areas that are marked with dashed lines as representing the absorption and emission by trimeric PBPs. In other words trimeric PBPs have been considered as the basic building blocks of the heterogeneous rods and cores.

e. Depending on the results and clues obtained on APC core in our studies on Nostoc rivulare as well as also from the informations gathered on APC core from other organisms, we assume that the core complex has both short wavelength and long wavelength emitters.

In this scheme, energy transfer within the pigment bed of an individual PBP has been described by introducing S, M and F chromophores. These chromophores differ in their spectral behaviour (Grabowski & Gantt, 1978; Mimuro et al, 1986;
Glazer, 1983). Further, the scheme also demonstrates that the probability of absorption of green-orange light (545nm) decreases with decreasing energies along the energy transfer pathway. Thus, the energy absorbed by the PEC in the heterogeneous rod, is subsequently passed onto the next pigment-protein PC before ultimately it reaches to the APC core.

The transfer process in the core complex appears to be more complex and may be realized in several steps. Our experimental results have shown that the short wavelength emitters, particularly APC II, are present in relatively much higher amounts than the long wavelength emitters (APC I & B). In such situations, we may assume that energy migration from rods to core through short wavelength emitters (APC II & III) predominates over the one via long wavelength emitters. However, there is strong possibility that at least a significant percentage of energy may be transferred to either of the two or both the long wavelength emitters within the core complex from where, it ultimately migrates to terminal emitters (large molecular weight polypeptides or coloured bilin containing polypeptides). This leads to the completion of entire process.