High-Resolution Crystal Structures of Trimeric and Rod Phycocyanin

Liron David†, Ailie Marx† and Noam Adir*

Schulich Faculty of Chemistry, Technion—Israel Institute of Technology, Haifa, 32000 Israel

The phycobilisome light-harvesting antenna in cyanobacteria and red algae is assembled from two substructures: a central core composed of allophycocyanin surrounded by rods that always contain phycocyanin (PC). Unpigmented proteins called linkers are also found within the rods and core. We present here two new structures of PC from the thermophilic cyanobacterium Thermosynechococcus vulcanus. We have determined the structure of trimeric PC to 1.35 Å, the highest resolution reported to date for this protein. We also present a structure of PC isolated in its intact and functional rod form at 1.5 Å. Analysis of rod crystals showed that in addition to the α and β PC subunit, there were three linker proteins: the capping rod linker (LR8.7), the rod linker (LR), and only one of three rod–core linkers (LRC, CpcG4) with a stoichiometry of 12:12:1:1:1:1. This ratio indicates that the crystals contained rods composed of two hexamers. The crystallographic parameters of the rod crystals are nearly identical with that of the trimeric form, indicating that the linkers do not affect crystal packing and are completely embedded within the rod cavities. Absorption and fluorescence emission spectra were red-shifted, as expected for assembled rods, and this could be shown for the rod in solution as well as in crystal using confocal fluorescence microscopy. The crystal packing imparts superimposition of the three rod linkers, canceling out their electron density. However, analysis of B-factors and the conformations of residues facing the rod channel indicate the presence of linkers. Based on the experimental evidence presented here and a homology-based model of the LR protein, we suggest that the linkers do not in fact link between rod hexamers but stabilize the hexameric assembly and modify rod energy absorption and transfer capabilities.

Introduction

The phycobilisome (PBS) is an enormous (3–7 MDa) light-harvesting complex made up of pigmented phycobiliproteins (PBPs) and mostly unpigmented proteins (collectively termed linker proteins) that are arranged in two highly organized subcomplexes: six to eight rods surrounding a core of two to five cylinders.†–5 Models of the specific arrangement of the rods surrounding the core to form the overall PBS architecture have been suggested on the basis of electron microscopy of isolated PBS particles, stabilized in high-ionic-strength phosphate buffer. It is clear that the direction of energy transfer correlates with the position of the pigment within the complex. The higher energy-absorbing phycoerythrin (λmax =560 nm) or phycoerythrocyanin (λmax =575 nm) are found at a distal position in the rods of some species, followed (in all species) by phycocyanin (PC; λmax =620 nm), which always
occupies rod positions proximal to the core containing allophycocyanin (APC; \(\lambda_{\text{max}}=650\) nm).\(^1\) The initial building block of all PBPs is a heterodimer formed by the association between \(\alpha\) and \(\beta\) PBP subunits, commonly called an \((\alpha\beta)_2\) monomer. Three monomers readily associate to form an \((\alpha\beta)_3\) trimer, the most stable form of isolated PBPs in the absence of high concentrations (>0.5 M) of phosphate. Four APC trimers (including minor variants \(\beta'^{16}\) and \(\alpha'^{16}\)) associate to form core cylinders, and two to five cylinders further assemble in the presence of the L\(_{\text{CM}}\) linker protein into the core subcomplex. The core is associated with the thylakoid membrane and is closest to the membrane-bound photosystems. Two PC, phycoerythrin, or phycoerythrocyanin trimers assemble into hexamers through contacts made by \(\alpha\) subunits and hexamers assemble further into rods (through contacts made by the \(\beta\) subunits). The rods and core contain at least seven different linker proteins, and their assembly into the rods or core cylinders may require the activity of molecular chaperones.\(^7,8\)

The trimeric aggregation of the PBPs leaves a central hole with a diameter that ranges between 25 and 50 Å, and this is proposed to be the location of the linker proteins.\(^9\) Linkers have three proposed roles in the PBPs: (i) organization and stabilization of the separate PBP subcomponents in the entire PBP complex, (ii) determination of the direction and termination of the rods and method of attachment of the rods onto the core, and (iii) modification of the spectral properties of the PBPs, by modifying the individual chromophore environments. Based on the particular position occupied in the rods, linker proteins are categorized into four classes:\(^10\) rod linkers (L\(_R\)), rod–core linkers (L\(_{RC}\)), core linkers (L\(_C\)), and core–membrane linkers (L\(_{CM}\)). There are four different L\(_{RC}\) linker types (encoded by genes cpcG1–cpcG4\(^{11,12}\)) grouped according to sequence homology,\(^12,13\) although not all species contain all four L\(_{RC}\) types. There have been suggestions that each type has a different role in PBP stabilization and that different PBPs types may exist.\(^12,14\) CpcG1 has been proposed to be the major L\(_{RC}\) linker, required for PBP stabilization, while CpcG2 has been proposed to be present in smaller PBPs aggregates serving as an antenna to PSI.\(^14–16\) CpcG3 and CpcG4 are shorter and more similar to each other,\(^13\) but no specific role has been suggested for these linkers to date. CpcG4 has been shown to be present in isolated PBS from Anabaena sp. PCC 7120\(^{12}\) while CpcG3 was shown to be present in PBS isolated from Mastigocladus laminosus.\(^17\)

X-ray crystallography has provided excellent molecular descriptions of the isolated structures of PBPs.\(^1,8,18–27\) Crystallization of the PBPs in most cases has been quite straightforward, due to these proteins’ solubility characteristics as well as their propensity to self-assemble into extended structures. However, in the process of their isolation, PBPs rapidly lose their association with one another and with the linker proteins. The relatively hydrophobic linker proteins have remained poorly characterized structurally, and only a single-crystal structure of an intact linker exists—that of the L\(_C\) linker crystallized within APC trimers [Protein Data Bank (PDB) code 1B33]. The presence of this small linker was shown to impart a flattening of the trimeric ring that alters the distances between subunits. However, the authors of this study concluded that the complete internalization of the linker with the ring would appear to negate its postulated role as a capping protein. In addition, the two linkers in the asymmetric unit had variable positions and high B-factors, indicating weak or variant binding.\(^27\) Two crystal structures of phycoerythrin were obtained from hexamers that were shown to contain the \(\gamma\) linker subunits, but these could not be visualized in the electron density as a result of threefold averaging used during structure refinement.\(^28,29\) Recently, the NMR structure of a single domain of the L\(_{CM}\) linker from Anabaena was deposited in the PDB (code 2KY4), showing a compact six-helical bundle.

PC structures can be grouped into two types, those that form hexamers but not rods \emph{in crystal} and those that form hexamers and rods \emph{in crystal}. PC from Thermosynechococcus vulcanus and Thermosynechococcus elongatus\(^{18,19,23}\) belongs to the latter group; however, a unique PC was isolated from \(T.\) vulcanus and shown to be a blue-shifted (PC\(_{612}\)) form,\(^20\) which does not associate into hexamers or rods \emph{in crystal}. This crystal structure was obtained from a novel fraction of PC with an unmethylated Asn\(_{72}\) residue and was proposed to be a minor PBS component, a trimeric form of PC that may form contacts between rod and cores. The PC structures that contain hexamers \emph{in crystal} include structures from thermophilic cyanobacteria, mesophilic cyanobacteria, and red algae.\(^{19,21,22}\) In the structures of PC from \(T.\) vulcanus, \(T.\) elongatus, and Fremyella diplosiphon, the hexamers pack directly one on top of the other, in identical orientations. These rod-forming hexamers also associate laterally \emph{in crystal}, with each rod surrounded by six adjacent rods.\(^1\) In other PC structures, the hexamers do not form rods; rather, they are arranged in layers or at right angles. A result of this heterogeneity in crystal packing is that it is reasonable to question the validity of the proposal that rods \emph{formed in crystal} are equivalent to those found in the PBS \emph{in vivo} as visualized by transmission electron microscopy measurements.

We can best address issues pertaining to the functionalities of the PBS, particularly in terms of assembly and energy transfer, by crystallization of isolated components that afford high-resolution structure determination coupled with attempts to stabilize and crystallize larger complexes. Very high
resolution structures of the PBPs can elucidate structural subtleties essential for mapping out functionalities. In this respect, structures that could possibly resolve the positions of hydrogen atoms would be particularly useful, since the protonation state of the cofactors and their protein/solvent surroundings are critical in fine-tuning the absorption and energy transfer aspects of light-harvesting pigments. Structural characterization of the entire PBS complex or the subcomplexes (rods or cores) is necessary to elucidate the overall architecture of the complex and resolve questions such as the mechanism of attachment of rods to the core, the arrangement of rods around the core, and the possible interactions between laterally associated rods. One final aspect that is needed to complete the picture is a structural description of the linker proteins that occupy the central cavity of the PBPs. In this report, we show progress in both of the experimental directions described above: we present a structure obtained from PC preserved in rod form (Tv-Rod), and a new high-resolution structure of trimeric PC (Tv-PC). The biological relevance of rods formed in crystal during the crystallization of isolated PBPs is discussed, and a new model for the sequestering of the linkers is proposed.

Results and Discussion

High-resolution PC structure from T. vulcanus

In order to improve the diffraction quality of crystals of isolated trimeric PC, we collected numerous diffraction data sets from crystals [grown from isolated (αβ)$_3$ trimers] obtained in a range of crystallization and cryoprotection conditions. The crystals that exhibited diffraction to the highest resolution (better than 1.3 Å) were obtained in the presence of 0.8 M ammonium sulfate containing 10.5% sucrose. As for the previously determined PC structures from T. vulcanus obtained from crystals grown in polyethylene glycol (PEG) 4000, the new structure contains a monomer in the asymmetric unit cell, which represents one-sixth of a hexamer from which infinite rods are formed during crystallization. The structure refined to final $R_{work}$ and $R_{free}$ values of 21.0% and 22.6%, respectively, with excellent geometry [Table S1; RMSD of 0.5 Å (α chain) and 0.8 Å (β chain) for all atoms with the previously determined 1.6 Å 1KTP structure]. The electron density is excellent over the entire model, including the three phycocyanobilin cofactors (Fig. 1), and the average B-factor is 20.9 Å$^2$. At this resolution, minor or alternative side-chain conformations become visual. For instance, two conformations were observed for the αGln57, which forms a symmetry-related salt bridge linking two adjacent rods in the crystal (Fig. S1). βAsn148, which is on the outer circumference of the trimer ring, also has multiple orientations.

Tv-PC crystal structures from a wide range of crystallization conditions are invariant

Over the past 25 years, PC has been crystallized from different organisms and under different crystallization conditions. Comparisons between...
different structures are useful in order to achieve higher confidence in the relevance of the structures to the actual state of the protein and cofactors in solution. However, it is clear from many studies that crystallization can impart at least two types of structural modification on the protein. The crystal lattice is obtained by the formation of intermolecular interactions that, in many cases, are not of a physiological nature. These interactions can induce modifications to the protein structure such as changes in side-chain rotamer position, changes in external loop orientations, or even significant movement of entire domains. A second source of structural changes caused by crystallization can result from the protein’s environment during crystallization (precipitant type, presence of salts, cryoprotecting agents, etc.). Since it is our goal to obtain the highest degree of chemical understanding of the PC structure, it is important to determine which structural facets are independent of crystallization. In the case of PC structures, the appearance of rod-like structures in crystal has been suggested as being related to the forces needed to assemble the PBS rods seen in electron microscopy studies. This is not necessarily so, since the linker proteins are absent in all PC structures to date, while it has been shown that mutant strains lacking rod linkers are devoid of rods.32,33 In addition, it is not clear whether higher levels of crystal packing (between adjacent rods) are physiologically relevant.

Our previously determined structure of trimeric PC at 1.6 Å (IKTP) was obtained in the presence of PEG 4000, at pH 7.0, and the crystals were treated with silicon oil prior to flash freezing.31 In order to obtain even higher-resolution details on PC, we improved our isolation procedures (see Materials and Methods) and embarked on a systematic screen of numerous crystallization and cryoprotection conditions. Among the many trials performed, we found that crystallization in ammonium sulfate and use of sucrose as the cryoprotectant (introduced during crystallization or prior to flash freezing) consistently produced superior results and resulted in the improved resolution as already described above. In addition to these conditions, PC crystals were also obtained in other conditions (Table S2). In all, we determined five PC structures from different crystallization and cryoprotection conditions at better than 2 Å. All crystals of trimeric PC were found to belong to the same space group (H32, with a single monomer in the asymmetric unit) with essentially identical unit cell dimensions as those obtained for PC crystals grown from PEG solution and identical crystal packing interactions. This rather unique situation, where a protein crystallizes in exactly the same habit, under significantly different conditions, permits us to identify the effect of precipitants (salts versus polymers) and/or cryoprotectants on the orientations of side chains, on the structure of the cofactor, and on the positions and number of water molecules identified in the electron density maps. Table S2 shows that the position of all protein and cofactor atoms in all structures determined are equivalent, within the coordinate error of determination obtained by calculation of Luzzati plots. The major difference between the structures is in the number and positions of solvent water molecules. It is of particular interest to note that in every PC trimer structure solved here, as well as the PC rod structures (see below), a water molecule was found bridging the propionic acid groups of the phycocyanobilin cofactor attached to the αCys84 residue. All structures but one (PS_AS_EG, Table S2) also had a similar water molecule intervening between the two propionic acids of β84 phycocyanobilin, while in the β135 phycocyanobilin, the water content was more variable. The possible structural significance of this water molecule has been discussed previously18,19 and these current results certainly support this. The isomorphous nature of the PC structures over a wide range of crystallization conditions may indicate that the contacts formed here are indicative of true biological contacts and not a mere result of the crystallization process. Assessment of the biological validity of equating the PC rod structures identified in crystal with rods formed in vivo was one of the reasons to pursue the possibility of obtaining crystal structures of higher-order subcomplexes.

Isolation, characterization, and crystallization of PC rod substructures

All published procedures to isolate either the entire PBS or subcomplexes (rods or cores) require the presence of high concentrations of phosphate (or citrate) buffers at near-neutral pH.34 This requirement is most likely due to the need to replace some characteristic of the membrane-bound PBS in vivo, that is, the presence of unknown small molecules in the cytoplasm and/or the effect of protein crowding.35–41 In the process of isolating the entire PBS by sucrose gradient centrifugation, we obtained bands that contained a higher PC-to-APC ratio (than for intact PBS), indicating the presence of free rods. These rods were purified on a 0.5- to 0.8-M sucrose gradient (in 0.5 M phosphate) as detailed in Materials and Methods. SDS-PAGE analysis of the purified complex (Fig. 2, lanes 1) showed the presence of all rod components in the presence of core contaminants. Due to the absolute requirement for the high-ionic-strength phosphate buffer to preserve rod integrity, further purification by ion-exchange-based chromatographic methods was precluded. However, although the solution of isolated rods was heterogeneous, crystals were obtained in the presence of high phosphate buffer. The crystals
were morphologically similar to Tv-PC crystals in terms of shape and color, and thus, we performed further analysis on the crystals themselves in order to verify their constituent components. Crystals were collected and washed extensively in high phosphate buffer in order to remove possible remnants of non-crystallized protein, and they appeared translucent with no visible precipitated amorphous material. The protein composition of these crystals was determined by mass spectrometry (Table 1) and SDS-PAGE (Fig. S2), which confirmed the presence of the α and β PC subunits in the presence of three different linker proteins: the LR 8.7 rod capping linker, the LR rod linker, and only one type of LRC rod–core linker (cpcG4). Densitometric analysis of the SDS-PAGE of solubilized crystals shows that the ratio of the rod components was 12:12:1:1:1 (Table S3), which is consistent with a two-hexamer rod. This is the same ratio of rod linkers to PC subunits obtained from analysis of isolated intact PBS (Fig. S2 and Table S3).

Functional intactness of the rod was confirmed by spectroscopic analysis (Fig. 3). Both the absorption and fluorescence emission spectra of linker-

![Table 1. Composition of Tv-Rod crystals determined by mass spectrometry analysis](image)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Gene</th>
<th>Function</th>
<th>Identified peptides (%)</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-PC</td>
<td>cpcA</td>
<td>PBP</td>
<td>89</td>
<td>49</td>
</tr>
<tr>
<td>β-PC</td>
<td>cpcB</td>
<td>PBP</td>
<td>78</td>
<td>51</td>
</tr>
<tr>
<td>LR</td>
<td>cpcC</td>
<td>LR</td>
<td>37.5</td>
<td>21</td>
</tr>
<tr>
<td>LR 8.7</td>
<td>cpcD</td>
<td>LR 8.7</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>LRC</td>
<td>cpcG4</td>
<td>LRC</td>
<td>29</td>
<td>17</td>
</tr>
</tbody>
</table>

* The number of identified peptides out of the number of possibly identifiable peptides resulting from trypsin cleavage expressed as a percentage.
* The number of residues identified from the total number of residues in the protein expressed as a percentage.

![Fig. 2. SDS-PAGE analysis of isolated rods. Lanes 1 and 2, isolated rod fraction components prior to and following cross-linking with GA, respectively. (a) PBPs visualized by Coomassie brilliant blue stain. (b) PBPs visualized by intrinsic fluorescence under UV illumination. M₀, molecular weight marker (Precision Plus Protein, Bio-Rad).](image)

![Fig. 3. Spectroscopic analysis of Tv-PC (solution) and solubilized Tv-Rod crystals. (a) Room temperature absorption spectra and (b) fluorescence emission spectra (excitation, 580 nm) of isolated Tv-PC (dotted line) and Tv-Rod (continuous line) in the presence of 0.5 M phosphate.](image)
associated PC have been well characterized in previous studies\textsuperscript{42–44} and are distinct from that of trimeric PC. Rods have an absorption maximum at 629–638 nm, red shifted compared to that of trimeric PC whose maximum is at 621 nm,\textsuperscript{4,43} and this was confirmed in the rods isolated here, with an absorption maximum at 635 nm (Fig. 3a). Similarly, the expected red shift in the fluorescence emission spectra\textsuperscript{42,46} was obtained when the rod sample was excited at 580 nm (Fig. 3b). In order to confirm that all linkers are in close proximity to the PC rod disks rather than simply being present in the solution, we performed cross-linking experiments. SDS-PAGE analysis of isolated rods after cross-linking with glutaraldehyde showed that the original linker bands were absent and replaced by new bands of higher molecular weight (Fig. 2, lanes 2). The intrinsic fluorescence of the PC subunits was used to identify which of the new bands contained PBPs, and indeed, all of the bands resulting from crossed linked bands contained PBPs, and the apparent molecular weight indicated products of cross-linking of both PC subunits (α or β) and each of the linker proteins (as well as higher-order products). Each band was excised from the gel and analyzed by mass spectroscopy to show the exact subunit composition (Table S4), revealing that all of the linker components cross-linked to the PBPs. Bands of free linkers or linkers cross-linked only to other linkers were not observed.

Absorption spectroscopy analysis of solubilized Tv-Rod crystals showed the expected maximum at 635 nm as was already measured for rods prior to crystallization. To further assure the presence of the linkers within the crystallized rods, the fluorescence emission spectrum of a single crystal was obtained by direct examination using confocal fluorescence microscopy (Fig. 4), which enabled the analysis of the fluorescence at different positions and depths (spatial resolution of 1 μm) of the crystal. The crystal was excited by a DPSS laser (561 nm), and the resulting emission of the single Tv-Rod crystal showed a peak at 655 nm at all positions and depths. For comparison, a crystal of Tv-PC was similarly analyzed and exhibited an emission peak at 649 nm. Both crystals show a slight red shift from their respective emission peaks in solution (Fig. 3). The red shift in both Tv-PC and Tv-Rod single-crystal emission spectra indicates that higher-order oligomerization of PC (in crystal) affects PC’s spectral characteristics, even when linkers are absent, but the presence of the linkers induces a further red shift.

Structure determination of \textit{T. vulcanus} rods

Several structures from the Tv-Rod crystals were determined in the space group \textit{H32}, with unit cell dimensions nearly identical with those of the Tv-PC. The highest-resolution structure was at 1.5 Å, and the final model was refined to an $R_{\text{work}}$ and $R_{\text{free}}$ of 23.9% and 26.0%, respectively. The RMSD for all atoms between the Tv-Rod and the Tv-PC structure is 0.4 Å. Since the isolated rods crystallize in a manner identical with that of trimeric PC, infinitely elongated rod assemblies are formed, with each rod containing one 3-fold and two 2-fold rotational axes (Fig. S3). The symmetry axes run directly through the rods, and thus, the linkers that occupy the central cavity are positioned directly on these axes. The three linkers in the rod crystals are thus physically superimposed, resulting in weak and disorganized density. Recently, single-particle electron microscopy analysis of PBS isolated from \textit{Synechocystis} appeared to show densities representing the linker proteins in the center of isolated rods.\textsuperscript{47} The density appears to be divided into three smaller masses. This observation is not easily explained since each hexamer contains no more than one full L_{H} or L_{RC} linker protein (see below) and the rod linker proteins do not have any apparent repeating units (on the basis of their amino acid sequence) that would easily form three identical masses. It is however clear from the results presented here that the linkers present in the rod crystals do not affect crystallization and, thus, most likely do not project out from the rod channel as previously proposed.\textsuperscript{9,46} This is also the case for the linker found in phycoerythrin hexamers\textsuperscript{28} and the L_{C} linker within an APC trimer, as visualized in the 1B33 structure.\textsuperscript{27}

Potential linker interactions in the Tv-Rod structure

Since we could not build an experimentally based molecular model of the linkers within the Tv-Rod structure, we queried whether the presence of linkers in the crystallized rod has an effect on the Tv-Rod
structure that can be identified. Small patches of electron density resulting from the presence of a linker protein have been noted in several structures of phycoerythrin. These structures were also solved from proteins that crystallized in space groups with 3-fold symmetry: P3 or H3. In two cases, the authors assigned the small patches of electron density to either a single amino acid or to short peptides putatively belonging to the linker. In both cases, the identifiable electron density was in the vicinity of the cofactor facing the inner cavity. In the PC-Rod structure presented here, we do not see identifiable linker residues. Four PC residues that project into the hexamer cavity are stabilized significantly, based on their improved electron density: αGlu7, αArg15, βLys7, and βGln113. All of these residues are in positions that could interact with the C-terminal domain of either LR or LR8.7 based on their homology to LC and to the position of LC in the 1B33 APC structure.

The crystallographic B-factor gives a measure of the flexibility of an atom about an average position. It is reasonable to assume that a significant reduction in the B-factor of residues exposed to the central cavity in the Tv-Rod structure might provide indirect evidence that the side-chain conformation is stabilized by the presence of the linkers. Since all three rod linkers are superimposed, only those residues that interact with all three linkers should be significantly stabilized. The average B-factors for the Tv-Rod and Tv-PC structures are similar, 20.6 Å² and 20.9 Å², respectively; however, differences in the specific B-factors for some individual atoms and residues showed significant variance between the two structures. Atoms where the B-factors are larger by at least 20 Å² in one structure compared to the other were identified, and the relative position of

Fig. 5. Calculated electrostatic potential of the hexamer surfaces. The inner surface of the hexamer central cavity (one-third of a hexamer is shown).

Fig. 6. Structural model of the LR linker. (a) Sequence alignment of LR from T. vulcanus and the structurally characterized component of LCM from Anabaena sp. (underlined with a bold line, 2KY4) and LC from M. laminosus (underlined with a dotted line, 1B33 subunit N). (b) Three-dimensional model of the full-length LR built using the I-TASSER protein modeling server. The model is based on the two independent domains PFAM00427 (2KY4, blue cartoons) and PFAM10383 (1B33 N subunit, red cartoons) structures.
the residues is shown in Fig. S4. It is of note that there were fewer atoms identified as having significantly higher B-factors in the PC-Rod structure, and in these cases, the atoms were often main-chain atoms. While the identified residues are positioned variously throughout the PC monomer and clearly the B-factor difference in most cases is not the direct result of linker interactions (but could be the result of the overall effect of the presence of the linker), αGlu109 is a residue of particular interest since it projects out into the rod cavity and has significantly lower B-factors in the Tv-Rod structure when compared to the Tv-PC structure. In the 1B33 structure, which contains the LC linker, the homologous αGlu105 residue on one of the three APC monomers interacts with the C-terminus of the LC linker (Fig. S5) and has a lower B-factor than the same Glu residues on the two monomers that do not interact with LC. The most prominent peak is that of βArg79, which has a much higher B-factor in Tv-Rod than in Tv-PC. This residue is involved in trimer formation and does not face the inner hexamer channel.

It is interesting to note that the electrostatic surface potential of the inner face of the hexamer (formed by six copies of the α and β subunits) is composed of patches of charged regions (on the top and bottom circumference) with a more hydrophobic area in the central circumference (Fig. 5). On the other hand, the rod linkers are hydrophobic and are almost completely insoluble in isolated form. 46,52 Sequence homology studies on linkers suggest that the LR and LRC linkers contain two domains: a small C-terminal domain (PFAM 01383)53 that is homologous with both LC (found in the 1B33 structure) and LRC and a larger N-terminal domain (PFAM 00427). The N-terminal domain of LR is homologous to a PFAM 00427 domain of LCM recently determined by NMR (PDB code 2KY4). We have used the I-TASSER protein structure prediction engine54 to build a homology-based model of the LR linker based on these two structures (Fig. 6). This folding engine returned models with relatively low confidence levels, mostly due to the lack of overlap between the two domains and thus lack of confidence in their relative positions. The RMSD for the PFAM 00427 domain of the model and the 2KY4 structure is 0.8 Å, while the models of the PFAM 01383 domain are less similar to the 1B33 linker (5–8 Å). From the determination of the Tv-Rod structure presented here, it appears that the linkers do not project out from the rod cylinder. The combination of the linker hydrophobicity and the PC polarity would appear to indicate very loose association between the two components. This is supported by the propensity of these components to separate upon isolation.

Given that the probable location of the linker proteins is within the confined cavity created by the association of PC monomers into trimers and hexamers, it is of interest to estimate the free volume within this space and the extent to which this volume may support loose PC-linker association. The volume of the cavity created in a hexameric aggregation of PC was calculated using the 3V:Voss Volume Voxelator server as 74,100 Å³ (Fig. 7). Presuming that a rod is composed of at least two hexamers, this means that the three linker proteins LR8.7 (molecular mass=8.7 kDa), LR (molecular mass=32 kDa), and LRC (molecular mass=30 kDa) can occupy a volume at least twice this size. Given an average protein density of 1.35 g/cm³, the volume occupied by these three linkers should be of the order of 86,000 Å³, which is only about 60% of the available space in the central cavity of a two-hexamer rod. The LR and LR8.7 models have calculated volumes of 37,000 and 13,700 Å³, respectively, which agrees with the above estimation. These calculations suggest that the cavity is spacious enough to support significant freedom of movement, which would be consistent with the lack of strong interactions between the linkers and PBPs.

Since the Tv-rod crystals contain only a single LRC linker type (CpcG4, Table 1), it is possible that we
have crystallized only one form of rod and that the other rods indeed have protruding \( L_{RC} \) linkers, which might inhibit crystallization. The *T. vulcanus* PBS contains six rods surrounding the tri-cylindrical core. A number of models have been suggested for the manner of rod assembly onto the core.\(^{1,5,11}\) The model that is supported by the crystal structures of isolated components depicts the six rods attached to the core in a fashion that there are three rod doublets.\(^{1,2,5,11}\) Two doublets extend out parallel with the thylakoid membrane and each rod interacts directly with the circumference of a core cylinder. One doublet projects out in a direction perpendicular to the other two doublets. Each of the two rods associates with both the outside of a rod from one of the lower doublets and with a single core cylinder. These rods may not be attached to the PBS in the same fashion as the two doublets parallel with the membrane, and thus, their \( L_{RC} \) may be totally hidden within the rod. These may be the rods that we have successfully crystallized; however, only further study on the whole PBS will enable us to ascertain whether there are indeed different rods at different positions surrounding the core.

**Model for linker association with rods and cores**

It has been generally assumed that the linkers actually link hexamers, by extending out from one hexamer to the next. There is actually little clear-cut evidence for this suggestion,\(^{2,9,45,57}\) but it is clear that in mutants lacking a specific linker, the appropriate substructure (rod or core) does not assemble correctly. In all electron microscopy studies published,
visualized masses that have been proposed to be linkers do not protrude from the subunits, perhaps due to the low resolution of these studies. Phycocyanthin hexamers have been crystallized with the internal linker, but it could not be crystallographically visualized, appearing to indicate that the linker is totally sequestered in the cavity. This is the case for the \( \text{L}_\text{C} \) linker in APC as well. In fact, the four APC trimers that make up each core disk are held together without the presence of an intervening linker protein, since the \( \text{L}_\text{C} \) resides in a single trimeric disk and the \( \text{L}_{\text{CM}} \) linker domains are proposed to reside outside the discs. Our cavity volume measurements show that a PC hexamer has an empty volume of about 74,000 Å\(^2\) and that an \( \text{L}_\text{R} \) will take up about 37,000 Å\(^2\) and the \( \text{L}_8 \) will take up an additional 13,700 Å\(^2\). Thus, there is ample room for both linkers to reside in a single, distal hexamer. We propose here a new model for the linker proteins within the rods, since the \( \text{L}_\text{C} \) resides in a single trimeric disk and the \( \text{L}_{\text{CM}} \) linker domains are proposed to reside outside the discs. Our cavity volume measurements show that a PC hexamer has an empty volume of about 74,000 Å\(^2\) and that an \( \text{L}_\text{R} \) will take up about 37,000 Å\(^2\) and the \( \text{L}_8 \) will take up an additional 13,700 Å\(^2\). Thus, there is ample room for both linkers to reside in a single, distal hexamer. We propose here a new model for the linker proteins within the rods, since the \( \text{L}_\text{C} \) resides in a single trimeric disk and the \( \text{L}_{\text{CM}} \) linker domains are proposed to reside outside the discs.
Biochemical and spectroscopic analysis of Tv-Rod solution and crystals

Cross-linking experiments were performed on the isolated rods in solution with 0.5% glutaraldehyde for 30 min at 4 °C. Rod solutions before and after the cross-linking were run on SDS-PAGE and unstained gels were also visualized by UV illumination. Quantitative densitometric digitations and analysis of SDS-PAGE stained with Coomassie brilliant blue were performed using Gel Analyzer 2010†. The gel background was subtracted using the rolling-ball method.

Analysis of the Tv-Rod crystal components was achieved by collecting five to six crystals from the mother liquor of the crystallization drop and washing them thoroughly and repeatedly before resuspension in water. Tv-Rod crystals solubilized in water were characterized by SDS-PAGE and mass spectrometry using a Tandem RPLC-ESIMS (Somler Proteomics Centre, Technion—Israel Institute of Technology). The fluorescence emission spectra from single crystals were obtained using an LSM 510 META laser scanning confocal microscope (Zeiss). The fluorescence emission spectra from single crystals were obtained using an LSM 510 META laser scanning confocal microscope (Zeiss) with a DPSS laser with excitation line at 561 nm. The focus was reduced to a spatial resolution of 1 μm, and the crystals were typically 10–50 μm thick (in the direction of the exciting beam).

Calculation of the volume of the PC hexamer cavity and linker models

The volume of the PC cavity and linker models was determined using the 3V:Voss Volume Voxelator server.55

For the cavity calculation, an outer probe size of 25 Å and an inner probe size of 3 Å were used, and for the volume calculations, a probe size of 3 Å was used.

Accession numbers

Coordinates and structure factors have been deposited in the PDB with accession numbers 3O18 and 3O2C for the Tv-PC and Tv-Rod structures, respectively. Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2010.10.036

Acknowledgements

This work was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (1045/06). We gratefully thank the staff of the ESRF (beamlines ID-14-1, ID23-1) for provision of synchrotron radiation facilities and assistance.

References

212 Crystal Structures of Trimetric and Rod Phycocyanin


