

Crystal Structure of a Light-Harvesting Protein C-Phycocyanin from *Spirulina platensis*

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Received February 28, 2001

The crystal structure of C-phycocyanin, a light-harvesting phycobiliprotein from cyanobacteria (blue-green algae) *Spirulina platensis* has been solved by molecular replacement technique. The crystals belong to space group P2₁ with cell parameters $a = 107.20$, $b = 115.40$, $c = 183.04$ Å; $\beta = 90.2^\circ$. The structure has been refined to a crystallographic R factor of 19.2% ($R_{\text{free}} = 23.9\%$) using the X-ray diffraction data extending up to 2.2 Å resolution. The asymmetric unit of the crystal cell consists of two $(\alpha\beta)_6$ -hexamers, each hexamer being the functional unit in the native antenna rod of cyanobacteria. The molecular structure resembles that of other reported C-phycocyanins. However, the unique form of aggregation of two $(\alpha\beta)_6$ -hexamers in the crystal asymmetric unit, suggests additional pathways of energy transfer in lateral direction between the adjacent hexamers involving $\beta 155$ phycocyanobilin chromophores. © 2001 Academic Press

Key Words: crystal structure; C-phycocyanin; light-harvesting; phycobiliprotein; bilin; chromophore; *Spirulina platensis*.

Phycobilisomes are supramolecular complexes of phycobiliproteins, which constitute the major light-harvesting antennae for oxygenic photosynthesis in cyanobacteria (blue-green algae) and red algae. In phycobiliproteins, the chromophore, a linear tetrapyrrole (bilin) is covalently attached to the apoprotein by thioether bonds to cysteine residues. Allophycocyanin (APC), C-phycocyanin (CPC), phycoerythrin (PE) and phycoerythrocyanin (PEC) are the principal classes of phycobiliproteins in phycobilisome assembly. Their collective range of absorption spans the entire visible

spectrum of sunlight. The antennae rods of phycobilisomes are composed in the order of APC at the core, CPC in the middle and PE/PEC at the tip. The energy transduction proceeds in the direction from tip to the core through PE/PEC $\rightarrow$ CPC $\rightarrow$ APC $\rightarrow$ Reaction center, with an overall efficiency greater than 95% (1, 2).

Electron microscopic (3, 4) and crystallographic (5–16) studies have provided a wealth of information on structure–function relationship of phycobiliproteins. Previously determined crystal structures of phycobiliproteins, namely that of APC (5–7), PE/PEC (8–11) and of CPC (12–16) have revealed that the tertiary fold and the general architecture of macromolecular assemblages are remarkably conserved in all classes of phycobiliproteins. They consist of α - and β -subunit polypeptides, which exhibit high affinity for one another and associate into $(\alpha\beta)$ -monomers, which in turn aggregate into $(\alpha\beta)_3$ -trimers and $(\alpha\beta)_6$ -hexamers. Hexameric phycobiliproteins together with the linker proteins, self-assemble *in vivo* into macromolecular light-harvesting complexes (4).

The cyanobacterium *Spirulina platensis* contains only two phycobiliproteins, APC and CPC (17). CPC is the major light-harvesting pigment protein present in the antenna rods of *S. platensis*, whereas APC is a minor component present only at the core. The medicinal and pharmacological properties of CPC have been reported earlier (18). Recent studies have demonstrated the hepatoprotective (19), anti-inflammatory (18, 20, 22) and antioxidant (18, 21) properties of CPC. CPC is also used in photodynamic therapy of various cancerous tumors (23). In this paper, we present the crystallization and crystal structure of CPC from *S. platensis* (CPC_{SP}). Interestingly, the CPC_{SP} molecule crystallizes as two $(\alpha\beta)_6$ -hexamers in the asymmetric unit. The close association of adjacent hexamers provides a new possibility of exploring an additional role for $\beta 155$ phycocyanobilin (PCB) chromophore in

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terms of physical mechanism of light energy transfer in lateral direction.

MATERIALS AND METHODS

Materials. CPC (isolated from *Spirulina platensis*) was a generous gift from Cyanotec Bio-products (P) Ltd. (Bangalore, India). It was further purified as reported earlier (17) to a linker free form of CPC and was characterized by UV-visible spectroscopy, SDS-PAGE and electrospray-ionization mass spectrometry (21). All the reagents used during purification and crystallization were of analytical grade.

Crystallization and data collection. Blue colored crystals of typical size 1.0 mm × 0.5 mm × 0.1 mm suitable for X-ray diffraction analysis were obtained in about 3–4 weeks at room temperature by sitting drop vapor diffusion technique. The experimental condition consisted of 4 μ l drops of 30 mg/ml protein dissolved in water mixed with 3 μ l of the reservoir equilibrated against a reservoir containing 23% (w/v) PEG 6000, 10% (v/v) of ethanol, 75 mM of phosphate buffer (pH 6.8). Three dimensional intensity data were collected on a Quantum-4 CCD detector from a crystal cryocooled to 100 K using synchrotron radiation source at beamline X4A of National Synchrotron Light Source (NSLS), at the Brookhaven National Laboratory (BNL), U.S.A. The wavelength of incident radiation was 0.98 Å and the crystal to detector distance was 197.5 mm. With an oscillation range of 1.0° for each image, a total range of 154° was covered. Autoindexing routines gave solutions consistent with a monoclinic space group, which enabled unit-cell dimensions to be refined to $a = 107.20$, $b = 115.40$, $c = 183.04$ Å, $\beta = 90.2^\circ$. Data were processed in $P2_1$ space group using HKL2000 suite (24). The Matthew's coefficient V_m value (25) of $2.5 \text{ Å}^3 \text{ Da}^{-1}$ is consistent with the presence of two $(\alpha\beta)_6$ -hexamers, i.e., 12 $(\alpha\beta)$ -monomer aggregates in the crystal asymmetric unit. The solvent content is estimated to be 51%. In this calculation, the value of 0.74 cc/g was used as the protein specific volume.

Structure solution and refinement. The sequence of α and β chains of CPC_SP were retrieved from SWISS-PROT database with primary Accession Nos. P72509 and P72508, respectively. Based on high sequence homology, CPC from *Fremyella diplosiphon* (15), (CPC_FD) was chosen as a search model. The structure of CPC_SP was determined by molecular replacement using $(\alpha\beta)_3$ -trimer as a search model, generated from the coordinates of CPC_FD $(\alpha\beta)$ -monomer (protein data bank access code 1cpc) and using the program AMoRe (26). The best four solutions corresponding to a high co-relation coefficient of 63.3% and a R factor of 36.4% were used to generate the two $(\alpha\beta)_6$ -hexamer models of CPC_SP. The model coordinates were subjected to crystallographic refinement using the program CNS (27) with manual interventions of model modification using program O (28) and quality check using PROCHECK (29). The topology restraints for chromophores were obtained from the HIC-UP server (30) and applied during refinement. Initially, the refinement was carried out with noncrystallographic symmetry restraints between either all 12 $(\alpha\beta)$ -monomers or the two $(\alpha\beta)_6$ -hexamers of the asymmetric unit. However, inspection of relative change in R_{cryst} and R_{free} factors with and without noncrystallographic symmetry restraints led us to remove the restraints. The data collection and the refinement statistics are given in Table 1. The 12 $(\alpha\beta)$ -monomers, totaling to 24 chains were named in alphabetical order A, B, C, ..., X with alternate alphabets corresponding to α - and β -chains, respectively. The first 12 chains A to L correspond to one hexamer and chains M to X correspond to the second hexamer in the asymmetric unit.

RESULTS AND DISCUSSION

Description and quality of the structure. An estimation of the root-mean-square error is obtained by Luz-

TABLE 1
Summary of Crystallographic Analysis

Measurement	Value
Data collection	
Resolution limit (Å)	2.2
Total reflections	1177344
Unique reflections	223480
Completeness, %	99.3 (97.8)
R_{merge} , %	8.9 (44.5)
Refinement statistics	
Resolution (Å)	25–2.2
Number of non-H protein atoms	31,572
Number of water molecules	2,290
R_{cryst} , %	19.2 (26.2)
R_{free} , %	23.9 (30.3)
RMSD bond lengths (Å)	0.006
RMSD bond angles	1.135
Average B-factor (Å^2)	27.3

* Values in parentheses correspond to the last resolution shell from 2.28 to 2.20 Å.

zati plot (31) and suggests an upper limit of 0.24 Å for the CPC_SP model. The quality of the final model is summarized in Table 1. The inspection of the electron density of the model indicated a few modifications in the sequence. Residue $\alpha 51$ was changed from Arg to Leu. This is consistent with the multiple alignment of known sequences of CPCs where Leu at $\alpha 51$ is highly conserved. Inspection of extra density at $\alpha 148$ position prompted us to modify the existing residue Gly to Thr. Another modification was done in β -chain corresponding to Asn to Ser at position $\beta 78$. All the modifications were monitored by examining $(2F_o - F_c)$ omit maps at the corresponding regions, which also resulted in a significant decrease of R_{cryst} and R_{free} factors. Some of the long polar side-chain residues had relatively weak density. The most consistently disordered side-chains among all the chains were Lys $\alpha 47$, Arg $\alpha 120$, Arg $\beta 116$ -Glu $\beta 117$. Also, there is a segment of three residues (Gln-Met-Gln) corresponding to two of α -chains, M68-M70 and U68-U70, where electron density is weak. Side-chain density for Leu $\beta 120$, a hydrophobic residue occurring at the surface, indicates disorder. The (ϕ, ψ) values of nearly all non-glycine residues are within the allowed regions of Ramachandran Plot (32). One exception is noted for Thr $\beta 77$ ($\phi = 84.6^\circ$, $\psi = 143.8^\circ$), at the beginning of helix E (Fig. 1), which interacts through a hydrogen bond between its amide nitrogen and an oxygen of chromophore $\alpha 84$. The strained geometry of this residue is conserved in all known phycobiliprotein structures (5–16). Methylation of Asn $\beta 72$ residue at γ -N position could be clearly modeled into a visible $(2F_o - F_c)$ electron density map. This side chain is involved in providing crucial contacts through polar interactions to the chromophore $\beta 84$ (Table 2) and also isolating it from interactions with the solvent. Methylation of Asn $\beta 72$ is a highly conserved feature of all

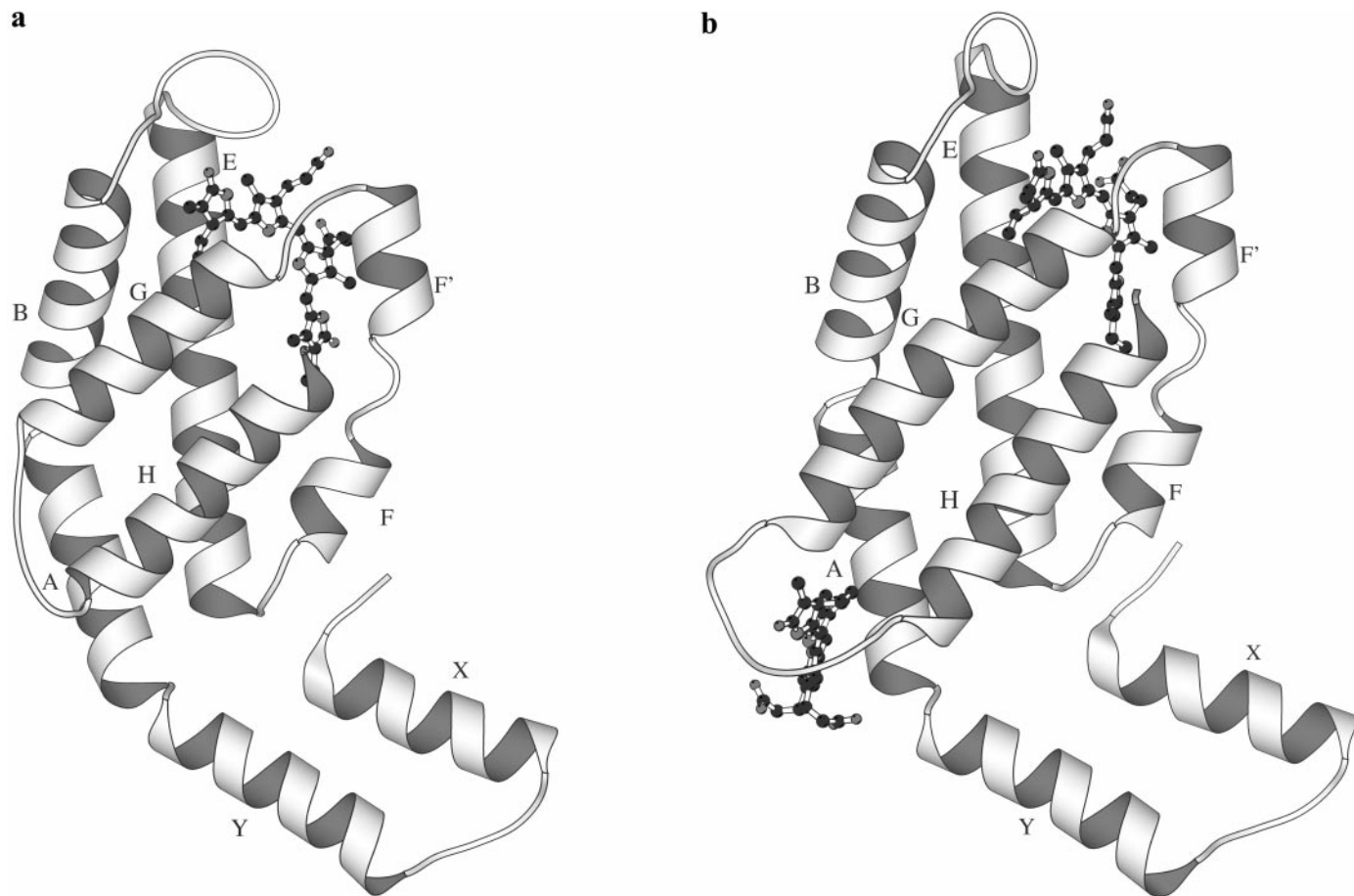


FIG. 1. Ribbon representation of (a) CPC_SP α -subunit (b) CPC_SP β -subunit. Chromophores are shown in ball and stick representation. Helices are named following the convention given in Ref. (12). This figure was prepared using the program MOLSCRIPT (37).

reported phycobiliproteins, without which the energy transfer is less efficient (33). It may be speculated that the structural location of methylated Asn β 72 might introduce a crucial change in the electronic property (16) of the microenvironment of β 84-PCB during the hexameric aggregation, thereby influencing the energy transfer rates.

The chain fold of the molecule is predominantly α -helical (Fig. 1) and is similar to the known structures of CPCs, which is expected from the high level of sequence identity between them. The overall fold of each chain is a well-defined, helical globin-like domain with seven helices (A, B, E, F, F', G and H) folded similar to that of myoglobin. The PCB chromophore attachment sites at α 84 and β 84 positions correspond to the heme-group binding site of myoglobin (34). The globin domain is complemented by an additional helical hairpin domain (X and Y) at the N-terminus of each chain, which is responsible for the stability of $(\alpha\beta)$ -monomers (12). These similarity features seen in the present structure are in agreement with the earlier reports of distant evolutionary relationship between globins and CPCs (12, 34). Further, the α - and β - polypeptides of

CPCs were predicted to be related to each other based on hydrophobicities of amino acid sequences (35). The evolutionary relationship between them is evident from the remarkably similar tertiary fold of the two chains in CPC_SP (Fig. 1) and in other phycobiliproteins (5–16). The structural superposition of CPC_SP α - and β -chains shows two regions of residue insertions with respect to each other. The first one corresponds to a two-residue insertion in α -chain at position 73–74 extending the loop region between B and E helices (Fig. 1a). The second insertion corresponds to a ten-residue stretch in β -chain. This occurs between the residues 146–155 in the loop region between the helices G and H, corresponding to a functional accommodation of an additional chromophore at β 155 (Fig. 1b).

Each of the $(\alpha\beta)$ -monomers has high affinity to assemble together to form a $(\alpha\beta)_3$ -trimer. The $(\alpha\beta)_3$ -trimers associate in back to back fashion to form a $(\alpha\beta)_6$ -hexamer, the functional unit of a phycobilisome assembly. The highly symmetrical aggregation of monomers into hexamers involves molecular 32-symmetry (12). The coincidence of molecular symmetry with the crystal symmetry results in the occurrence of

TABLE 2
Protein–Chromophore Polar and Ionic Interactions for α - and β -Subunits

	α 84*	β 84*	β 155*
OA	Asn 73A O 3.52 Ala 75A N 3.14	Men† 72B OD1 3.49	Gly 153B O 3.26 Gly 153B N 3.24
N21	Asn 73A O 3.15	Men† 72B OD1 3.10	Thr 151B O 2.92
N22	Asp 87A OD2 2.81 Tyr 129A OH 3.48	Asp 87B OD2 2.89	Asp 39B OD2 2.75
O1B	Arg 57F NH1 2.85 Wat 10 OH2 2.71 Pro 72A O 3.58		Asn 35B OD1 2.89
O2B	Wat 204 OH2 2.70 Wat 1071 OH2 2.69	Arg 77B NH1 2.68 Arg 77B NH2 3.26	
O1C	Arg 86A NH1 2.87 Arg 86A NH2 3.19 Wat 248 OH2 2.64	Arg 86B NH1 3.26 Arg 86B NH2 2.81	Wat 1119 OH2 2.54 Thr 151B OG1 2.83
O2C	Wat 204 OH2 2.63 Lys 83A NZ 2.68		Wat 840 OH2 2.94 Thr 151B OG1 3.46
N23	Arg 86A NH2 2.96 Asp 87A OD2 2.74 Asp 87A OD1 3.12	Asp 87B OD2 2.89 Arg 86B NH2 3.66 Asp 87B OD1 3.14	Asp 39B OD2 2.60 Wat 1433 OH2 3.34
N24	Wat 248 OH2 3.07 Wat 797 OH2 3.49		Asp 145G OD1 2.94
OD	Thr 77F N 3.11 Wat 797 OH2 2.86		Asp 145G OD1 3.10 Gln 33G NE2 3.31

* Entries in these columns are ordered as residues, position number, chain id, atom name, and distance in Å.

† Men residue stands for γ -N-methylasparagine.

monomeric or dimeric ($\alpha\beta$) aggregates in the crystal asymmetric unit as seen in earlier structures (7–16). The present structure forms a unique example of hexameric phycobiliprotein occurring in the asymmetric unit of a crystal system without utilizing crystallographic rotational symmetry elements and there are two such hexamers (Fig. 3) in the asymmetric unit. The overall hollow cylindrical structure of CPC_SP ($\alpha\beta$)₆-hexamer resembles closely the unit cell constructs of other phycobiliproteins. The outer diameter of the hollow cylinder is approximately 110 Å with a height of about 60 Å. The two hexamers are slightly offset with respect to the crystallographic *b*-axis. The axis passing through the center of hexamer1 is tilted by about 7° to the *b*-axis, while it makes an angle of 87° and 83° with *a*- and *c*- axes respectively. In comparison, the central axis of hexamer2 is less tilted at about 3° from the *b*-axis while making an angle of about 88° each with *a*- and *c*- axes. Further, the angle between the central axes of two hexamers is 5°. These subtle deviations from exact symmetry between the two ($\alpha\beta$)₆-hexamers could be the reason for CPC_SP crystallizing in a lower symmetry crystal system compared to other phycobiliproteins.

Chromophore structure and environment. High quality of the electron density has allowed us to model all atoms of the chromophores unambiguously (Fig. 2) in all the chains. All together, 36 PCB chromophores were located, 3 chromophores for each ($\alpha\beta$)-monomer

at α 84, β 84, and β 155 positions. All the PCBs are covalently attached to the respective cysteine residues in protein by thioether linkages. The average B-factors for the PCBs are 22.6 Å² except for PCB attached to β 84 of T chain for which it is 44.5 Å². The conformation of PCB is similar to that seen in the model structure of CPC_FD (15). The stereoisomers for PCB at C₃₁ atom of ring A (Fig. 2) are (R) for α 84, β 84 and (S) for β 155. All chromophores display Z configuration on the D ring as found in CPC_FD (15). The polar and ionic interactions (Table 2) also depict highly conserved environs of the chromophores. These networks of interactions are responsible for maintaining the proper orientation of the chromophores. This in turn stabilizes the light absorption properties and energy transfer rates of each of the chromophores.

Energy transfer. The energy transfer rate between two chromophores is strongly dependent on the relative distance and orientations of the chromophores (36). Distances between the various PCBs were calculated as between the centroids of π -conjugated parts of a chromophore. Comparison of distances between the chromophores within a hexamer of CPC_SP with that of CPC_FD and other CPCs (12–16) shows that the transfer mechanisms are similar in nature. It was shown that energy transfer between hexamers in an antenna column takes place predominantly with α 84 and β 84 PCB chromophores, with peripheral β 155 PCBs playing relatively less significant role (13–15).

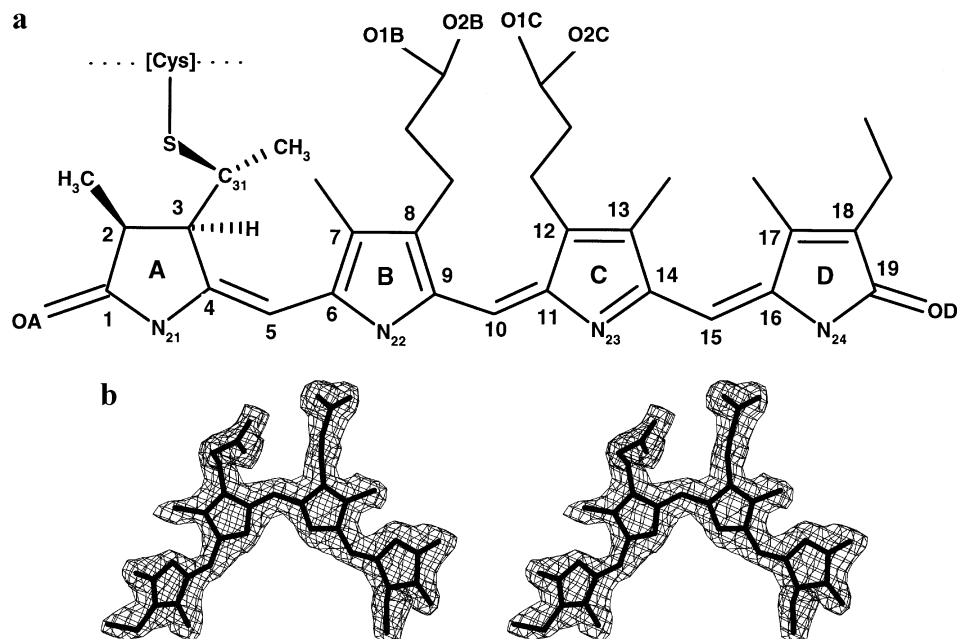


FIG. 2. (a) Chemical structure of PCB chromophore with the atom labels used in the discussion. (b) Stereo view of (2F_o-F_c) electron density map displayed at 1.0σ level for a β84-PCB chromophore.

As an outcome of the “double hexameric” aggregation in the present structure, β155 PCBs are brought to a close proximity of each other within the region between the adjacent hexamers in the crystal asymmetric unit (Fig. 3). The respective β155 PCBs and relative distances between them are summarized in Table 3. This suggests an additional possible role for β155 PCBs in terms of energy transfer in lateral direction between adjacent disks of CPCs. This could possibly be impor-

tant considering the *in vivo* environs of CPCs. Electron microscopic studies (3,4) have inferred that *in vivo*, CPC along with its linker proteins has a role of bridging the APC core with PE/PEC tip of the phycobilisome antenna rod. Near the bridging region where several antenna rods of CPCs come in contact with APC, it is likely that the CPC disks from adjacent rods come close to each other in a fashion similar to that seen in the CPC_SP crystal structure. Thus, the present crystal

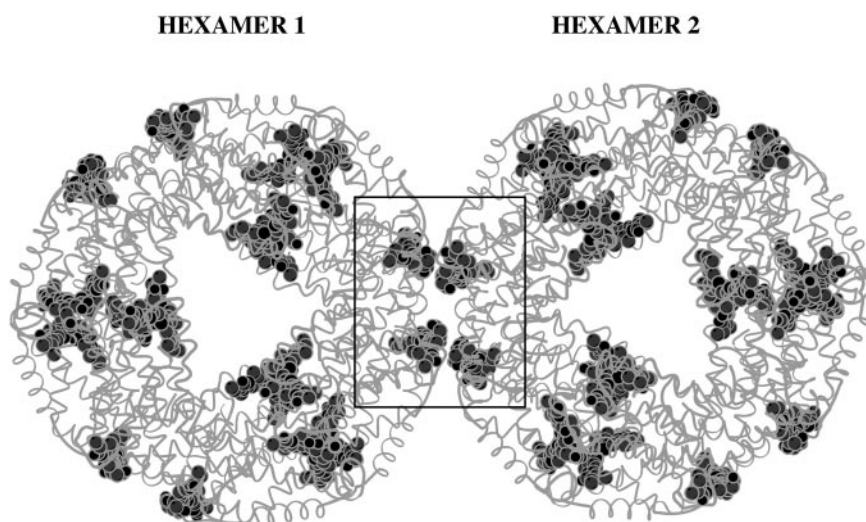


FIG. 3. Coil representation of the two hexamers in the crystal asymmetric unit, a view through the approximate central axis of hexamers. Chromophores are shown in CPK representation. The figure illustrates the arrangement of chromophores at various locations within the hexamers. The box drawn at the center highlights the close proximity of β155 PCBs in the region between the adjacent hexamers. This figure was prepared using the program MOLSCRIPT (37).

TABLE 3

Distances (in Å) between the β 155 PCB Chromophores of Adjacent Hexamers in the Asymmetric Unit of the Structure

	Hexamer 1		Hexamer 2	
	B β 155	H β 155	R β 155	V β 155
B β 155	—	28.14	29.81	21.86
H β 155	28.14	—	21.34	20.40
R β 155	29.81	21.34	—	27.98
V β 155	21.86	20.40	27.98	—

Note. Distances were calculated as between the centroids of π -conjugated parts of chromophores.

structure of CPC, the major light-harvesting pigment protein in *Spirulina platensis*, provides a possible model for lateral energy transfer between the adjacent CPC hexameric disks near the core region of native light-harvesting antenna rods.

ACKNOWLEDGMENTS

We are grateful to Cyanotec Bio-products (p) Ltd., Bangalore, India, for the supply of CPC_SP. We thank Dr. Craig Ogata for beamtime at beamline X4A, NSLS, BNL, U.S.A. and Dr. Z. Dauter for useful discussions. The financial support from DST, CSIR, and JNCASR, India, is acknowledged. We acknowledge the computational facilities at SERC, IISc and DBT supported Bioinformatics Centre, and Interactive Graphics Facility, IISc, Bangalore, for access to the resources.

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