Energy transfer dynamics in B-phycoerythrin from the red alga *Porphyridium purpureum*

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**Abstract**

The B-phycoerythrin hexamer \((\alpha\beta)_{6}\gamma\) of *Porphyridium purpureum* was isolated and purified. The absorption, circular dichroism, fluorescence and ultrafast time-resolved spectra were obtained. The results showed a double absorption peak at 545 nm and 565 nm and a shoulder peak at 498 nm, and fluorescence emission maxima at 580 nm and 620 nm were observed. The circular dichroism spectra in the near-ultraviolet region were obtained and resolved for the first time, which showed that the two peaks at 260 nm and 305 nm were considered to be correlated to phenylalanine (Phe) and tryptophan (Trp) in a conservative hydrophobic microenvironment, respectively. The circular dichroism spectra in the visible region showed that PEB139\(\alpha\)/PEB158\(\beta\) and PEB82\(\alpha\)/PEB82\(\beta\) existed as two exciton-coupled bilin pairs. Energy transfer within the exciton-coupled pairs was by exciton splitting, while between the exciton-coupled pairs was by Förster resonance. From the studies of the energy transfer dynamics by ultrafast time-resolved fluorescence spectroscopy, it was confirmed that the energy transfer of the B-PE hexamer had three time components of 8 ps, 60 ps, and 1200 ps. In addition, the internal energy transfer pathways of B-phycoerythrin hexamer were identified by deconvoluting the fluorescence decay curve at different detection wavelengths.

1. Introduction

Phycoerythrin (PE) is a kind of phycobiliproteins (PBPs) that exists in certain single-celled cyanobacteria that live under certain light conditions and almost all in red algae. They exhibit strong absorption in the green region of the visible spectrum from 480 nm to 570 nm and dense fluorescence emission at approximately 575 nm [1]. These red proteins can be divided into three subgroups, according to the absorption spectra distinguished by their structural specificity. These subgroups are B-phycoerythrin (B-PE; maximum absorption peak at 545 nm and a shoulder peak at 495 nm), R-phycoerythrin (R-PE; maximum absorption peaks at 565, 545 and 495 nm), and C-phycoerythrin (C-PE; maximum absorption peaks at 563 or 543 and 492 nm) [2-5]. The basic structural unit of PBP is a monomer \((\alpha\beta)\) with an \(\alpha\) subunit and \(\beta\) subunit, and the monomer further self-assembles into a trimer \((\alpha\beta)_{3}\). The trimer is a disc with a diameter of 11 nm, thickness of 3 nm and a central hole with a diameter of 1.5–5 nm. The basic structure of PE for energy...
transfer is a hexamer \((αβ)_6\), which is formed by ‘‘face-to-face’’ accumulation of two \((αβ)_3\) trimers. The basic structure of B-PE is \((αβ)_6\) and a γ subunit containing a unique chromophore [6-8].

B-PE is one of the most representative PEBs in red algae, which is mainly found in *Porphyridium cruentum*. *Porphyridium cruentum* is an unicellular red alga with a large star-shaped pigment body containing a large amount of PEBs, of which B-PE accounts for 42% of the total. In 1977, Glazer obtained B-PE from *Porphyridium cruentum* and found that B-PE contains three subunits, the molecular weight of the α and β subunits were 19.5 kDa, and the molecular weight of the γ subunit was 29 kDa. The total molecular weight of B-PE \((αβ)_6γ\) was 263 kDa, and it contained Phycoerythrobilin (PEB) and Phycourobilin (PUB) chromophores [9]. In 1987, Glazer studied the amino acid structure of B-PE and found that the α subunit had 164 amino acids and the β subunit had 177 amino acids [10]. Zeng Chengkui and co-workers isolated and purified B-PE and b-PE from *Porphyridium cruentum* in 1998 [11]. Comparing the results of electrophoresis between B-PE and b-PE, it was found that B-PE (containing the γ subunit) was much more stable than b-PE. Further studies indicated that the γ subunit had the function to stabilize the structure of PE, and the absorption shoulder peak at 498 nm was derived from the absorption of the PUB chromophore of the γ subunit. In 2012, Camara-Artigas et al. obtained the crystal structure of B-PE at pH 5 and pH 8, and obtained resolutions at 1.85 Å and 1.75 Å, respectively. From the crystal structure, it was further clarified that the structure of B-PE was a ‘‘disc’’ molecule with holes in the center, with the γ subunit rivets in the central cavity. The ‘‘disc’’ of the trimers consisted of three \((αβ)\) monomers surrounding the crystallographic cubic axis, and two ‘‘discs’’ were joined face-to-face to form a hexamer. The α subunit and the β subunit each contained 8 α-helices and 6 β-folds; wherein the α subunit contained two phycoerythrobilin (PEB) chromophores (PEB82α and PEB139α) and the β subunit contained 3 PEBs (PEB50–61β, PEB82β and PEB158β); and each PEB chromophore was within a distance of 30 Å [12]. This provided a good basis for resolving the functions of B-PE. In addition, Camara-Artigas also found that the microenvironment formed by the hydrogen bond between PEB82α and His88α of the B-PE α-subunit will be changed at pH 8.0, and protonation of the N atom results in a red shift of the absorption peak at 560 nm and a new absorption peak at 574 nm [13]. As far as is known, the γ subunit (linker) of B-PE contains PUB and phycourobilin (PUB), and is generally considered to contain two PUBs and two PUBs. Although the previous studies have clearly resolved the B-PE structure of *Porphyridium cruentum*, the details of energy transfer of the B-PE with 34 chromophores have not yet been resolved.

PBPs is the main light-harvesting complex of red algae, cyanobacteria and part of cryptophyte. At present, Förster dipole-resonance energy transfer (FRET) and exciton coupling can be used to describe the energy transfer process in PBPs [14]. When PBPs transfer energy in FRET mode, energy can only be efficiently delivered to the nearest receptor molecule. For long-range energy transfer of multiple molecules, it must be transmitted to the target molecule through multiple point-to-point migration. Considering the dissipation of energy during the transfer process, the energy transfer mode is less efficient. However, in PBPs, the energy transfer efficiency can be as high as 95% or more [15]. For this reason, the way in which light energy is transmitted between multiple chromophores has been the core issue in studying the structure and function of PBPs. MacColl et al. used the cryptophycobiliproteins (PE545 and PC645) as materials to study the exciton coupling of PBPs by means of circular dichroism, fluorescence polarization spectroscopy and time-resolved spectroscopy. The results show that three pairs of exciton-coupled chromophore pairs can be formed in PE545 and PC645, two of which are in the monomer and one pair on the surface between the monomers [16, 17]. In recent years, based on two-dimensional photon echo spectroscopy, the results show that the energy transfer of the internal chromophores of cryptophyte phycobiliproteins may be in a network state linked by quantum correlation [18-23]. However, in red algae and cyanobacteria, it is unclear whether exciton coupling mechanism exists.

Although the previous studies have clearly resolved the B-PE structure of *Porphyridium cruentum*, the details of energy transfer of the B-PE with 34 chromophores have not yet been resolved. To gain a deeper understanding of the structure and function of B-PE, especially the B-PE spectral changes caused by the γ subunit, we studied the spectroscopy of high-purity B-PE hexamers from *Porphyridium cruentum* to initially explore the energy transfer kinetics inside the B-PE hexamer using ultrafast time-resolved spectroscopy and spectral unfolding techniques. We expected to obtain more details about the energy transfer process of the original process of photosynthesis, which is of great significance for revealing the energy transfer relationship between B-PE and the photosynthetic system on the membrane.

2. Materials and methods

2.1. Materials

The microalga *P. cruentum* were obtained from the Laboratory of microalga biotechnology, Yantai University, China. The alga was intensively cultivated in Hemerick culture medium as has been described previously [24, 25]. The culture medium and the biomass were separated by centrifuging (10,000 × g for 10 min). The pellets containing biomass were lyophilized to obtain the dry biomass.

2.2. Preparation of analytical grade B-PE

The extraction was carried out using the method of Bermejo et al. with some modification. Freeze-dried samples (5 g) were resuspended in 50 mL acetic acid-sodium acetate buffer (pH 6.0, 1 M) [26]. The slurry was mixed with a variable-speed stirrer at 300 rpm for 1 h and then the slurry was centrifuged at 10,000 × g for 5 min.

The crude extract was filtered through a PES hollow fiber membrane (GM2540F1073, GE Water &Process Technologies, Shanghai) with a molecular weight cut-off (MWCO) of 30 kDa, 50 kDa and 100 kDa (shear force 0.1 MPa, flow rate 300 cm²/min, inner diam. 0.8 mm, outer diam. 1.2 mm, 4 °C). Deionized water was added constantly until the permeate liquid showed no...
absorption at 260 nm and 280 nm. The retentate was concentrated and lyophilized to yield 0.20 g of a fluffy red solid which was stored 4 °C.

After dissolving 0.20 g of the lyophilized retentate in 10 mL of sodium phosphate buffer (pH 7.2, 20 mM), the solution was further purified with a SOURCE 15Q (GE, USA) anion exchange column (50 × 16 mm) on a fast protein liquid chromatography machine (FPLC, AKTA purifier 10, GE, USA). After washing with the same buffer, the column was eluted with a linearly increasing ionic concentration gradient of NaCl (from 0 to 0.5 M) at a flow rate of 2.0 mL/min. The eluate with red color was pooled and analyzed.

2.3. Analyses by SDS-page and absorption and emission spectroscopy

Electrophoresis was carried out using a 12% polyacrylamide slab gel with 0.1% (w/v) SDS and 5% polyacrylamide stacking gel. Protein bands were detected by staining with 0.2% (w/v) Coomassie brilliant blue R250.

Light-harvesting ability was monitored by absorption spectroscopy. Energy transfer ability was monitored by fluorescence spectroscopy. The secondary structure was monitored by circular dichroism (CD) spectroscopy. The fluorescence spectra of B-PE were obtained by a fluorescence spectrophotometer (LS55, PerkinElmer, USA) at room temperature, with the final concentration of B-PE being 0.01 mg mL\(^{-1}\), and spectra were obtained with a path length of 1 cm, slit width of 5 nm, and excitation wavelengths of 280 nm, 360 nm, and 545 nm. The characteristic absorption peak was detected using a TU-1900 ultraviolet-visible spectrophotometer (Beijing Pu’s General Instrument Co., Ltd., China), where the scanning range was 400–750 nm, optical path length was 1 cm and step size was 1 nm, and the final protein concentration was 2 mg mL\(^{-1}\). CD spectra were measured with a J-180 circular dichroism spectrometer (JASCO Corporation, Japan), where the optical path length was 1 cm.

2.4. B-PE hexamer ultrafast time-resolved spectroscopy and fitting

Our experimental setup for time-resolved spectroscopy measurements is shown in Scheme 1. Femtosecond coherent light pulses at the wavelength of 525 nm were generated by an OPA system pumped by a Ti:Sapphire Amplifier (Legend Elite USP HE+, Coherent). This system produces pulses with a duration of about 35 fs, an average power intensity of 0.3–3 mW and a repetition rate of 1 KHz. Time-resolved fluorescence was detected by a synchroscan streak camera (Hamamatsu model C4742, time-resolution less than 1 ps) coupled to a polychromator. Thus the spectral and temporal characteristics of the sample fluorescence were recorded. Samples were held in a fused-silica flow cuvette. A total of 15 ml sample solution was recirculated through the sample cuvette, with the sample reservoir held at the temperature of 77 K.

The fluorescence detected by the streak camera was transferred into a computer for deconvolution. The detected fluorescence intensity \(F_{ex}(t)\) can be described as follows:

\[
F_{ex}(t) = \int_0^\infty F_t(t - \nu)I(\nu)\,d\nu
\]

Here we considered the theoretical fluorescence intensity \(F_t\) as a sum of multi-exponential form:

\[
F_t(t) = \sum_{i=1}^n T_i \exp\left[-t/\tau_i\right]
\]

We applied a deconvolution procedure based on a global optimization algorithm to fit the fluorescence.

3. Results and discussion

3.1. B-PE purity test

After purification by SOURCE 15Q chromatography, the electrophoresis results were consistent with previous reports [27, 28]. A broad band between 18 and 20 kDa (α and β subunits of B-PE) and a band at 31 kDa (γ subunit of B-PE) were observed from SDS-PAGE electrophoresis (Fig. 1). The above results indicated that the samples purified by SOURCE 15Q ion exchange chromatography
did not contain other proteins, the purity was higher, and B-PE maintained the natural conformation and stabilization of the structure and function of B-PE, by reducing the purification time.

3.2. Absorption spectroscopy and circular dichroism spectroscopy analysis of B-PE

Absorption and circular dichroism spectroscopy of the purified B-PE were performed, as shown in Fig. 2. The results of a full-wavelength scan of the B-PE sample showed that the hybrid protein peak (allophycocyanin at 620 nm) disappeared and B-PE purity ($A_{545}/A_{280}$) reached 5.1. The absorption spectrum of B-PE (blue line) exhibited three absorption peaks at 498 nm, 545 nm and 565 nm. Camara-Artigas et al. (2012) reported that there are three kinds of subunits that constitute B-PE of *P. cruentum* (α, β and γ subunits), wherein the α subunit is covalently bound to 2 PEBs, the β subunits are covalently bound to 3 PEBs, and the γ subunits are covalently bound to 2 PEBs and 2 PUBs [13]. The absorption peaks of B-PE at 545 nm and 565 nm were derived from the absorption of PEB, and the absorption peak at 498 nm was derived from the absorption caused by PUB.

The CD spectrum (red line) of B-PE exhibited multiple peaks and absorption intervals, which were at 260 nm, 305 nm, 330–380 nm, 498 nm, 518 nm, 543 nm, 563.5 nm, 576.5 nm and 590 nm. This was different from the absorption spectrum of B-PE. CD is a special absorption spectroscopy method that records the ellipticity at different wavelengths, which is very sensitive to the conformation of the chiral molecule, so it can reflect the finer structure of the chromophore. According to the energy level of the electronic transition, the circular dichroism of the protein is usually divided into three wavelength regions. Below 250 nm is the far-ultraviolet spectral region, which is where the CD spectrum mainly reflects the peptide bond structure of proteins, such as α-helices and β-strands. Since the crystal structure of B-PE has already been clearly determined, we focused on the near-ultraviolet region (250–300 nm) and the visible region (300–700 nm) of B-PE. In the near-ultraviolet region, the peak of the circular dichroism is mainly caused by the electronic transition of the aromatic group in the protein side-chain. In the visible region, a peak from circular dichroism is caused by a chromophore as the protein prosthetic group.

First, in the near-ultraviolet region of the B-PE CD spectrum, there was a positive peak at 260 nm and a negative peak at 305 nm. It was reported that circular dichroism of tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) exhibited peaks in the near...
ultraviolet region, in an asymmetric microenvironment. Phe shows weaker but sharper peaks at 255 nm, 260 nm and 270 nm, Tyr exhibits peaks at 275 nm and 282 nm, and Trp results in characteristic peaks at 290 nm and 305 nm. The CD spectrum of B-PE exhibited obvious positive and negative peaks at 260 nm and 305 nm. Consideration of the three Phe and one Trp of the α subunit and the five Phe of the β subunit in B-PE, the near-ultraviolet region CD spectrum of B-PE indicated that Phe and Trp may be co-located in a hydrophobic or asymmetric microenvironment. PE exhibited an absorption range of 330–380 nm in the visible light region, and there was a very strong positive peak. This was consistent with the absorption peak of B-PE, which was in the absorption range of the PEB and PUB chromophores.

Although B-PE contains only two PBPs chromophores, PEB and PUB, it has a broad absorption range from 498 nm to 590 nm and exhibits multiple absorption peaks. Since the absorption of phycobilidin in the visible region depends not only on its structure, but also on the surrounding proteins and amino acids, the optical properties of the PEB and PUB chromophores will vary in different protein domains [29].

In B-PE, the γ subunit contains PUB chromophores, while the α and β subunits contain only one PEB [9, 30]. Unlike PEB, PUB has extra double bonds on the A and B rings, so PUB can absorb shorter wavelength light than PEB. Thus the γ subunit can broaden the absorption spectrum of Porphyridium cruentum by PUB and absorb more light energy [31]. The γ subunit is located in the center of (αβ)3, while the γ subunit can avoid absorbing too much light and protect the photoreaction center of Porphyridium cruentum [32-35]. In accordance with recent research progress, 498 nm in the CD spectrum corresponds to the PUB chromodomain of the γ subunit. The B-PE fluorescence emission peak is usually at 580 nm, but the R-phycocyanin (R-PC) absorption peak is at 620 nm, which causes no light energy transfer between B-PE and R-PC through Förster energy resonance, because of the non-intersection between the emission peak of PE and the absorption peak of R-PC [1]. In 1998, the B-PE of Porphyridium cruentum was partially digested with proteinase K to isolate the native γ subunit, and its spectral properties and spatial position in the PE molecule were studied by Zeng Chengkui et al. [11]. The results of the γ subunit as the linker and enzymatic kinetic analysis indicated that the γ subunit was located in the central cavity of the B-PE hexamer (αβ)6. The absorption peak of PEB on the isolated γ subunit was at 589 nm, and the fluorescence emission peak was at 620 nm, which was related to R-PC. The overlapping of the absorption peaks contributes to the transfer of B-PE energy to the R-PC. In Fig. 1, we observed that the CD spectrum of B-PE exhibited a small shoulder at 590 nm, which was in agreement with the study by Zeng Chengkui et al. Thus, the shoulder at 590 nm in the CD spectrum corresponded to the PEB chromophore domain of the γ subunit.

To obtain a clearer CD spectrum image, we increased the concentration of B-PE within the detection range of the instrument and continued the measurement. The results are shown in Fig. 3. From the figure, we can see that the CD absorption peaks at 498 nm and 590 nm corresponded to the γ subunit of B-PE, exhibited no change and were clearer. However, a complex CD peak appeared in (αβ)6. Relative research on R-PE of red algae (Griffithsia monilis) indicated that when the PEB molecule heavily deviates from the conjugate plane (>50°), there is an absorption peak at 530 nm; when the PEB molecule slightly deviates from the conjugate plane Fig. 3. CD and absorption spectra (450–620 nm) of B-PE.
there is an absorption peak at 560 nm [34]. According to the crystal structure of B-PE, the pair of PEB82α and PEB82β exhibited lower off-plane properties than the pair of PEB139α and PEB158β, so the peak at 565 nm in B-PE absorption spectrum resulted from the pair of PEB82α and PEB82β, while the absorption peak at 545 nm was produced by the pair of PEB139α and PEB158β.

Camara-Artigas found that when the pH reached 8.0, the peaks of B-PE at 498 nm, 545 nm and 560 nm all disappeared, and a new absorption peak at 574 nm appeared. He speculated that the new absorption peak at 574 nm arose from the absorption peak redshifts, which were caused by protonation of His amino acid residues of PEB82α or PEB82β in high pH environments. The protein micro-environment of PEB82α or PEB82β is not only conserved in the red algae PBP, but also in cryptophyta [36, 37]. The micro-environment of PEB139α and PEB158β contains a conserved amino acid residue Asp, which maintains the chromophore micro-environment by a salt bond. When the pH increases, the salt bond dissociates, which results in the disappearance of the absorption peaks [13]. In this experiment, we found that even under normal physiological conditions, we could observe the absorption value at 576.5 nm. Additionally, the positive peak at 576.5 nm and the negative peak at 566 nm intersected at 568.5 nm which was near the absorption peak at 565 nm (produced by PEB82α and PEB82β). When two or more chromophores are close to each other and interact, the energy absorbed by the chromophore will be transformed into a high-energy state and a low-energy state, which interact in a strongly coupled environment, causing exciton splitting and excitation delocalization. One of the two CD peaks is a negative peak, and the other is a positive peak. The position at which the positive peak and the negative peak intersect usually corresponds to an absorption peak of the absorption spectrum. Therefore, the 576.5 nm and 566 nm absorption peaks in the B-PE CD spectrum corresponded to a set of exciton pairs. Since PEB82α is between the α subunit and the β subunit and PEB82β is in the middle of the hexamer, which is closer to the central γ subunit and can be used as the final energy emitter, the 576.5 nm peak in the CD spectrum corresponded to PEB82β, and the peak at 566 nm in the CD spectrum corresponded to PEB82α.

The positive peak at 543 nm and the negative 561.5 nm peak in the CD spectrum intersect at 556 nm, which suggests that there is also a set of exciton pairs. In *Porphyridium cruentum*, the structure of PEB139α in B-PE is different from that in other red algae [13]. PEB139α, Thr159β, and Asp157β form a chromophore microenvironment through hydrogen bonding, which results in the close distance of less than 20 Å for PEB139α and PEB158β in the hexamer. A similar structure also exists in the C-PC of Spirulina platensis, which allows light energy to be transferred laterally in C-PC [38]. It can be inferred from crystal structure analysis that PEB139α and PEB158β form another set of exciton pairs, which form the intersection of the positive and negative peaks at 543 nm and 561.5 nm.

From the CD spectra of B-PE and b-PE obtained by Glazer et al., the curves of B-PE at low concentrations were basically consistent with those shown in Fig. 2 [9]. After (αβ)6 of b-PE binds to one γ subunit, B-PE (αβ)6γ is obtained. In this process, the γ subunit affects the spatial structure of b-PE, and a new exciton pair appears (PEB82α or PEB82β), which creates a new intersection of positive and negative peaks near 568 nm, and the CD peak intensity near 545 nm drops sharply. The CD peak at 518 nm did not change, and a new CD peak appeared at 498 nm. Since the PUB and PEB absorption peaks of the γ subunit are located at the two ends of the B-PE absorption peak (498 nm and 565 nm), respectively, PUB causes the appearance of the 498 nm absorption peak, and PEB acts as the final acceptor of energy that interacts with PEB82β, which results in a new exciton pair of PEB82α and PEB82β. In B-PE, the γ subunit is located in the central cavity of the (αβ)6 cylinder, without intrinsic triple symmetry. Therefore, the γ subunit is unable to produce a symmetrical structural effect on PEB in (αβ)6. Thus, it is proposed that for the small peak at 531–537 nm (Fig. 3), the γ subunit tears the original PEB chromophore, which results in the changes of the original PEB chromophore structure domain. Referring to the structure and absorption peaks of DBV50–61β of PC-645, the CD peak at 518 nm should arise from PEB50–61β, owing to the evolutionary conservation of β subunit.

Fig. 4. Fluorescence emission spectrum of B-PE (λ<sub>ex</sub> = 545 nm).
3.3. Fluorescence spectral analysis of B-PE and discussion on energy transfer pathway

Purified B-PE (A545/A280 > 5.1) was excited at 545 nm and the fluorescence emission spectrum results are shown in Fig. 4. In the figure, the emission peak of B-PE was located at 575 nm, which was consistent with the reported of Tang et al. [39]. However, there was a very weak fluorescence emission peak at 620 nm. This should be the fluorescence emission peak of PEB of the γ subunit, and the energy emitted should be derived from the absorption at 590 nm in the CD spectrum (Fig. 1).

There were three distinct fluorescence emission peaks at 410 nm, 575 nm and 620 nm upon excitation at 360 nm (Fig. 5). The peaks of 410 nm and 575 nm were the combined effects of two close exciton pairs (PEB139α/PEB158β and PEB82β/PEB82α). The peak at 620 nm resulted from PEB in the γ subunit. The results of fluorescence emission indicated that B-PE can absorb the light energy by phycobilins and deliver it to R-PC.

The CD spectrum of B-PE has a positive peak at 260 nm and a negative peak at 305 nm, which resulted from the Phe-and Trp-in an asymmetric hydrophobic microenvironment. Meanwhile, the γ subunits were in the hydrophobic environment of the central cavity of B-PE. To test the possible energy transfer pathway between aromatic amino acids and γ subunits, we excited the aromatic amino acids at 280 nm. From Fig. 6, there were obvious fluorescence emission peaks at 303 nm and 620 nm, which were much higher than the two peaks at 330 nm and 410 nm; meanwhile, there was no high-intensity fluorescence emission peak at 575 nm (the high-intensity fluorescence emission peak at 560 nm was the frequency-doubled region of 280 nm).

The Trp will emit fluorescence at 348 nm upon excitation at 280 nm. Therefore, the weak fluorescence emission peak at 410 nm should arise from the low-energy chromophores with an absorption peak around 370 nm. Then, the low-energy chromophores receive the energy delivered by Trp. When the Tyr is excited at 280 nm, it emits fluorescence at 303 nm, which is just at the strong fluorescence emission peak in Fig. 6. By considering that there is a large amount of Tyr in B-PE, 10 Tyr in the α subunit and six Tyr in the β subunit, and B-PE has an absorption peak at 305 nm (Fig. 1), which overlaps with the absorption range of PUB in the near

![Fig. 5. Fluorescence emission spectrum of B-PE (λex = 360 nm).](image)

![Fig. 6. Fluorescence emission spectrum of B-PE (λex = 280 nm).](image)
ultraviolet, the energy absorbed by Tyr can be partially transferred to PUB. Therefore, PUB can emit fluorescence at 330 nm by absorbing a portion of the energy from Tyr emission, when the aromatic amino acids are excited at 280 nm. Thus the energy of the fluorescence emission of B-PE at 620 nm results from three phases, Tyr delivers energy to PUB, PUB absorbs energy, and Trp delivers medium energy chromatic energy. Since there is only one Trp in the (αβ) monomer of B-PE, but there are 16 Try, the emission peak at 410 nm is much lower than the emission peak at 330 nm. These results indicate that the energy absorbed by the aromatic amino acid can be transmitted to the γ subunit with higher efficiency, which also broadens the light absorption spectrum of Porphyridium cruentum and increases the usage efficiency of light energy.

3.4. Ultrafast time-resolved spectroscopy of B-PE hexamer

To further verify the internal energy transfer model of the B-PE hexamer (αβ)αγ proposed, we combined ultra-fast time-resolved spectroscopy with the spectral deconvolution procedure based on a global optimization algorithm. Using the time-resolved fluorescence spectrum of B-PE, a time-related fluorescence intensity–wavelength atlas was obtained at 77 K (Fig. 7). The delay time of the fluorescence spectra increased from top to bottom (from 0 to 240 ps, the interval between each two adjacent spectrum curves Δτ = 20 ps), with a pump laser wavelength of 525 nm and the detection range from 520 nm to 640 nm. The change of fluorescence spectrum in each time period could be visually observed from the atlas. In the initial stage, the emission peak was not visible until 40 ps, then it could be clearly seen that the B-PE hexamer exhibited a fluorescence emission at 546 nm. Combining the CD spectra and crystal data, this weaker fluorescence emission peak at 546 nm should be generated by the PEB chromophore that was torn from the γ subunit upon absorption of the excitation light energy. At the delay time of 80 ps, the B-PE hexamer shown the fluorescence emission at the wavelength of 575 nm.

This fluorescence emission peak at 575 nm was generated by two pairs of exciton pairs close to each other (PEB139α/PEB158β and PEB82β/PEB82α), by absorbing the emission energy of the PEB chromophore torn from the γ subunit; then, the energy of the fluorescence emission at 575 nm was transmitted to the PEB of the γ subunit, then passed downstream. The ultrafast spectrum from 40 ps to 80 ps corresponded to the energy transfer path in the above energy transfer model where the light energy oscillated between the two exciton pairs close to each other (PEB139α/PEB158β and PEB82β/PEB82α) in the central cavity of the B-PE cylinder and stayed on PEB82β (fluorescence emission at 575 nm), and finally transmitted the energy to PEB of the γ subunit.

To understand more about the spectral characteristics of the B-PE hexamers under low temperature conditions, we selected the time-resolved fluorescence spectrum at the delay time of 80 ps for fitting and deconvolution. Fig. 8 shows that the fluorescence emission peak of B-PE hexamer was mainly at 571 nm, which was basically consistent with the fluorescence emission peak (575 nm) at the delay time of 80 ps as can be seen in Fig. 7. Three spectral components, with peaks at 570 nm, 580 nm and 612 nm, were obtained by Gaussian deconvolution, which agreed well with the fluorescence emission spectrum curve with \( R^2 = 0.98173 \). The emission at 570 nm and 580 nm constituted the strongest emission peak of the two PEB exciton pairs at 575 nm; the lower energy emission peak at 612 nm may be consistent with the fluorescence emission peak at 620 nm in Fig. 4, which resulted from the γ subunit PEB chromophore of B-PE.

Therefore, the dynamic fluorescence attenuation at different wavelengths at 77 K was multi-exponentially fitted based on a global optimization algorithm (fitting curves in Fig. 9). The kinetic fluorescence spectra of the B-PE hexamers were decomposed at different detection wavelengths. The results are shown in Table 1. The component of the positive pre-exponential factor represented the fluorescence emission process, and the component of the negative pre-exponential factor represented the energy absorption process. The results showed that the kinetic fluorescence of the B-PE hexamers could be deconvolution with three indices, and three characteristic time constants were obtained: 8 ps, 60 ps and 1200 ps. Among the three characteristic time constants obtained, the time constant of 1200 ps was considered to be the average fluorescence decay lifetime of the end-receptor PEB chromophore for energy...
transfer, and the rest could be thought of as a function of the time constant of energy transfer.

Fig. 10 shows the delayed-amplitude correlation map (DAS) of the B-PE hexamer time-resolved fluorescence spectral unfolding results, which is based on Table 1. The time constant of 8 ps had a maximum amplitude at 550 nm. As the detection wavelength was increased, the amplitude gradually decreased. Then, at 570 nm, there was a negative amplitude, and the negative amplitude reached a maximum at 580 nm. This was the process of fluorescence growth, which indicated that the energy was transmitted from a chromophore that emitted at 550 nm to a chromophore that emitted at 570–580 nm; from the absolute value of the amplitude analysis, the relative efficiency of the energy transfer of components at this time was higher than that at the time constant of 60 ps. The time constant of 60 ps exhibited a maximum magnitude at around 570 nm, the negative emission amplitude was exhibited at 590 nm, which indicated the absorption behavior at this wavelength. Therefore, it was considered that energy was transmitted from a chromophore emitting at 570 nm to a chromophore emitting at 590 nm. The time constant of 1200 ps was approximately considered to be the average fluorescence decay lifetime of the PEB chromophore. From a comprehensive comparison of the CD and fluorescence spectroscopy results, both exhibited almost identical peak results. The energy transfer path corresponding to the time constant of 8 ps may be that the original PEB chromophore torn from the γ subunit absorbed the light energy of the excitation light for the first time, then the energy was transferred to the two exciton pairs (PEB139α/PEB158β and PEB82β/PEB82α) inside the central cavity of the (αβ)6 cylinder. The energy transfer path corresponding to the time constant of 60 ps may be that the two exciton pairs transferred energy to the PEB chromophore of the γ subunit.

Table 1
Deconvolution results of B-PE hexamer at different detected wavelengths.

| Eex/nm | Emn/nm | τ1/ps | A1/% | τ2/ps | A2/% | τ3/ps | A3/% |
|--------|--------|--------|------|--------|------|--------|------|
| 525    | 550    | 11     | 85   | 57     | 14   |        |      |
| 570    | 8      | −5     | 74   | 43     | 1043 | 56     |      |
| 8      | 60     |        |      |        |      |        |      |

Fig. 8. Deconvolution results of fluorescence spectra of the B-PE hexamer (77 K).

Fig. 9. Fluorescence decay fitting results of different probe wavelengths. Fluorescence decay curve (circle) and fitting results (red line) from excitation at (a) 550 nm and (b) 570 nm.
4. Conclusions

The B-PE was obtained after a two-step purification. The spectral detection data showed that B-PE has two absorption peaks at 545 nm and 565 nm, one shoulder peak at 498 nm, and fluorescence emission peaks at 575 nm and 620 nm. In B-PE, phenylalanine and tryptophan are in a hydrophobic and asymmetric microenvironment, and the energy absorbed by the aromatic amino acids can be transferred to the γ subunit of B-PE. Although phycobilin has the same optical rotation, after forming a microenvironment with amino acids, there are seven positive peaks (498 nm, 518 nm, 531 nm, 538 nm, 543 nm, 576.5 nm and 590 nm) and two negative peaks (561.5 nm and 566 nm), which showed a complex energy transfer path within the B-PE. In this article, based on the known B-PE crystal structure and other data, we initially inferred the internal energy transfer pathway of B-PE and established an energy transfer model. We also used ultrafast time-resolved spectroscopy techniques to verify and improve the partial transmission path of the B-PE energy transfer model, and obtained more detail about the energy transfer process inside the B-PE hexamer.

Combining the above results of the CD spectrum and ultrafast time-resolved spectroscopy, we propose an energy transfer model for B-PE (Fig. 11). After the PUB of the γ subunit absorbs light energy, the energy is transmitted to PEB50-61β. Then, the energy can be simultaneously distributed to the exciton pair of PEB139α and PEB158β, which has a similar structure and the spatial distance is less than 20 Å. Meanwhile, the above exciton pair is close to another exciton pair (PEB82α and PEB82β), which may produce quantum coherence. The light energy is linked between the four PBP chromophores, and the energy oscillates between the four PEBs, and stays on the PEB82β which is in the central cavity of the (αβ)6 cylinder. Then, the PEB82β transfers the energy to the PEB of the γ subunit inside the central cavity, and eventually the energy is transferred to the subsequent PBP b-PE and R-PC by the Förster energy resonance. However, it is well known that the delocalized state just after photo-excitation loses the quantum coherence due to the fluctuations by thermal dissipation, thus, it is difficult to determine whether the coherency of excitonic splitting in these dimer system is stable or not. Here in our experiment, we assumed the stability of the dimer system and the existence of stable exciton splitting/delocalization during CD spectra measurement. In order to further identify the excited state in the pigments from the peaks of our CD spectra results, the CD spectra detection under 77 K will be performed for our future work.

Although the research on the internal energy transfer of PC645 and PE545 from cryptophyte are becoming more and more perfect, the conformation, energy state, energy transfer pathway, especially the correspondence between chromophore and absorption spectroscopy of these chromophores have been controversial. As for B-PE, most of its energy transfer process stays in
speculative state. In recent years, two-dimensional photon echo spectroscopy has been used to measure protein molecules spanning 5 nm at room temperature and applied to photosynthetic bacteria light-harvesting systems containing 4000 pigment molecules[19]. In the future, we believe that this technology will provide a new method and research perspective for deep understanding of the energy transfer relationship between B-PE and the photosynthetic system on the membrane.

Author contributions

Song Qin and Mingyuan Xie initiated and supervised the project; Wenjun Li and Zhihong Tang prepared the samples; Yang Pu and Wenjun Li collected the spectral data and performed the spectral analysis; Zhihong Tang and Yang Pu performed the biochemical and biophysical analyses; Fuli Zhao and Mingyuan Xie analysed the ultrafast time-resolved spectroscopy, Song Qin wrote the manuscript with help from all authors.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgement

The authors gratefully acknowledge the financial support provided by the National Natural Science Foundation of China (grant numbers 41906109). This work was also supported by the Goverment of Guangdong Province for NSF (2016B0901918099), the Key Project of Coal-based Science and Technology in Shanxi Province(Grant No. FT2014-01) and the Scientific Research Foundation of Ludong University(grant number ly2014041).

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