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Three-stage refolding/unfolding of the dual-color β-subunit in R-phycocyanin from *Polysiphonia urceolata*

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Abstract

The conformational changes during refolding and unfolding of the dual-color β-subunit in R-phycocyanin (R-PC) were monitored by the spectra, fluorescence anisotropy, and FRET. It was observed that both of the refolding and unfolding of the β-subunit would undergo a three-stage conformational change, but in a reverse order. During the refolding process, at the first stage, the configuration of the tetrapyrrole chromophores transformed from the cyclohelical to the extended one, suggested by the blue-shifted spectra. At the second stage, recovery of the hydrogen-bond and hydrophobic interaction network fixed the chromophore in a more rigid configuration, suggested by a linear increase in the total fluorescence yield. At the third stage, the increase of the FRET efficiency suggested a protein-framework movement that made the two chromophores closer or/and into a more parallel orientation. The fluorescence anisotropy further confirmed the three-stage model.

Keywords: Dual-color β-subunit; Folding/unfolding; FRET; Conformation; Configuration; Protein-framework movement

How a protein folds from the random and disordered state to native one *in vivo* is one of the most challenging tasks facing biologists [1,2]. Study on the refolding of denatured polypeptides *in vitro* will provide a major insight. Up to now, various methodologies have been used to study on refolding/unfolding of proteins, including theoretical and experimental ones [3,4]. Among these, the spectral responses [5,6] and fluorescence resonance energy transfer (FRET) [7,8] are widely used as probes to monitor the conformational transition during the refolding/unfolding process of a protein. In this kind of studies, the intrinsic fluorophores [9–11] are preferred to the extrinsic ones [12], for the latter may introduce some structural modifications to the protein [13].

R-phycocyanin (R-PC) is a special kind of the light-harvesting pigment–protein complexes in phycobilisomes from some red algae. Different from C-phycocyanin (C-PC), which contains only phycocyanobilin (PCB) chromophores, R-PC contains both phycoerythrobilin (PEB) and PCB [14]. Recently, the crystal structure of a trimeric R-PC from *Polysiphonia urceolata* has been determined at 2.4 Å resolution [15]. In the trimeric R-PC, a monomer is composed of two nonidentical subunits, named α and β, with 162 and 172 residues, respectively. The α-subunit covalently links a PCB to the cysteine residue at α84 site, while the β-subunit links a PCB at β84 and a PEB at β155, respectively. In a native phycobiliprotein, the tetrapyrrole chromophores possess an extended configuration, while in the denatured protein the configuration of chromophores is supposed to be cyclohelical based on the spectral similarity to the free pigments [16]. Compared to the cyclohelical one, the extended configuration has a blue-shifted and narrower absorption band in the visible range [17]. In addition, the chromophores in the native protein possess rigid configurations so high fluorescence quantum yields, while in the denatured protein the chromophores are less rigid and nearly not fluorescent. Therefore, both

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of the spectral shift and the fluorescence fluctuation could be taken as probes to monitor conformational changes during the folding and unfolding of the protein. It is known that FRET depends on the distance, orientation, and spectral overlap of the donor to the acceptor [18]. The separation (36 Å) and orientation ($\theta \approx 37°$), calculated from the crystal structure data, as well as spectral overlap make the PEB (donor) and PCB (acceptor) in the β-subunit of R-PC form an ideal FRET pair. Any change of the distance and/or the orientation will certainly lead to variation of the FRET efficiency, which can be taken as an indication of the conformational transition of the subunit. In the past, R-PC has been studied mainly on their light-harvesting and energy transfer function. But in this work, the conformational changes during the folding/unfolding were studied by taking advantage of the dual-color β-subunit as a model. It was found that both of the refolding and unfolding of the β-subunit would undergo three-step conformational changes, corresponding to a transformation of the configurations, a modification of the microenvironments of the chromophores and the protein-framework movement, respectively, but in a reverse order.

Materials and methods

Materials. Ultrapure urea was purchased from Sigma. Bio-Rex 70 (400 meshes) was purchased from Bio-Rad Company. All other reagents of analytical grades were purchased from Beijing Chemical Plant (China). All working solutions were prepared immediately before use in redistilled water.

Separation of α- and β-subunits of R-PC. R-PC was isolated from the marine red alga *P. urceolata* collected on QingDao beach of East China and purified by the use of method reported previously [19]. The two subunits were separated by the procedures described earlier [20] with some modifications. All urea solutions used for separation of the subunits contained 1 mM β-mercaptoethanol and were adjusted to pH 3.0 with glacial acetic acid. First, 20 mg of lyophilized R-PC was dissolved in an 8 M urea buffer and stored for 1 h at −20 °C in the dark. The consequent thawed sample was diluted to 6 M urea and then applied to a column (1.5 × 20 cm) of Bio-Rex 70 (400 meshes) pre-equilibrated with 6 M urea. The successively eluted fractions at 6, 7, and 8 M urea were collected separately. The blue and the purple fractions eluted at 7 and 8 M urea are the α-subunit and β-subunit, respectively.

Renaturation of the β-subunit. The denatured β-subunit sample in 8 M urea, about 0.6 μM estimated by the absorbance and the reported ε_M values of 42,800 (at 561 nm) for PEB in acidic urea [21], was averagely separated into eight dialyzers (Spectra/Por 6 132572, molecular mass cut off of 10 kDa, Spectrum Medical Industries Inc.), and then dialyzed against 10 mM PBS containing 0.1 mM L-cysteine (1:1000 solution volumes) at 4 °C in the dark. Each sample was used to measure the absorption and fluorescence spectra at different time from 0.25 to 3.5 h after diluted to a constant volume with the synchronously dialyzed urea solution [22].

Urea-induced unfolding of the β-subunit. Equilibrium denaturation experiments were performed by mixing the appropriate volumes of renatured β-subunit stock (4 μM), buffer solution (10 mM PBS, pH 7.0), and 10 M urea stoke (containing 0.1 mM EDTA and 1.0 mM NaN_3), followed by incubation at 4 °C in the dark for 2 h to allow them to equilibrium. The consequent samples were used for the spectral measurements at room temperature. All of the working solutions were prepared immediately before use.

Spectroscopy measurements. Absorption spectra were obtained on a UV-1601 Ultraviolet/Visible spectrophotometer (Shimadzu, Japan) and fluorescence spectra on an F-4500 spectrofluorimeter (Hitachi, Japan). The excitation and emission slit widths were fixed at 5 nm and a square quartz cuvette of path length of 1 cm was used for fluorescence measurements. The emission intensities of PEB and PCB were monitored at 568 and 644 nm, respectively, with selective excitation of PEB at 540 nm. For fluorescence measurement, the maximum absorbance of a sample was kept no more than 0.1 to avoid any inner-filter effect. An FLS 900 spectrofluorimeter (Edinburgh Instruments, UK) equipped with polarizers was used for steady-state fluorescence anisotropy measurements with the slit widths fixed at 10 nm. The fluorescence anisotropy (r) was calculated from the following equation [23]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}. \quad (1)$$

Here, I_{VV} and I_{VH} are the vertically and horizontally polarized fluorescence components with excitation by vertically polarized light, and G, defined as I_{HV}/I_{HH}, is a sensitivity factor of the detection system. Each spectrum represents the averaged value of three times. The emission anisotropy values of PEB and PCB were monitored at 568 and 644 nm, respectively, with selective excitation of PEB at 540 nm at room temperature.

Results

The denatured β-subunit shows two absorption peaks in visible range at 561 and 663 nm while the renatured one at 550 and 618 nm, similar to those of R-PC trimer. The blue-shifted absorption spectra and the spectral similarity of the renatured subunit to the native R-PC suggest a correct refolding. Besides, the fluorescence recovery is also an indication of refolding, for the denatured subunit is almost not fluorescent. Specially, for the β-subunit, FRET from PEB to PCB was taken as a probe to monitor the refolding.

Refolding of the β-subunit

The absorption and the fluorescence spectra of the β-subunit as functions of dialysis time are shown in the insets of Fig. 1, from which the peak wavelengths (Fig. 1A and B) and the fluorescence intensities of PEB and PCB (Fig. 1C) are derived. The total fluorescence yields (Q_T) of the subunit, estimated as the relative areas under the curves of the inset in Fig. 1B, are shown in Fig. 1D. During the refolding process, three distinguishable stages could be recognized from Fig. 1. At the first stage, occurred roughly within the first 1 hour, the blue-shifted spectra (Fig. 1A and B), as well as the far lower fluorescence intensities (Fig. 1C) and yields (Fig. 1D), demonstrate a transformation of the configuration of the tetrapyrrole chromophores from the cyclohelical to the extended one. It was also observed that the peak shifts, an indication of the configuration transformation, occurred at around pH 3–4 (data not shown) which is similar to the pK value (3.86) of the carboxyl group in the side chain of a free aspartic acid (Asp). Therefore, formation of the salt bridge between the protonated N atom in PCB to the O atom in Asp87 or PEB to Asp39 may be a prerequisite to the extended configuration of chromophores [24]. At the second stage, roughly from 1 to 2.5 h, a monotonous increase in the fluorescence intensities (Fig. 1C) and the yields (Fig. 1D), as