Artificial chaperone-assisted renaturation of R-PE subunits

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Received (in Montpellier, France) 18th September 2002, Accepted 15th October 2002
First published as an Advance Article on the web 8th January 2003

The isolated α, β and two γ subunits of the R-phycoerythrin (R-PE) from *Polysiphonia urceolata* were renaturated by the artificial chaperone technique in combination with β-cyclodextrins. The molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are 17 580, 19 050, 32 500 and 34 060 for the α, β, γ_1 and γ_2 subunits, respectively. The renaturation yields of the four subunits obtained by the artificial chaperone technique are several times higher than those achieved by conventional methods. The two γ subunits are suggested to be naturally present species, instead of artificial products, considering the molecular weights and spectral features. The existence of two kinds of γ subunits may explain the failure to determine the entire structures of R-PE, b- or B-phycoerythrin (b- or B-PE) by X-ray crystal structural analyses.

Introduction

R-PE is a major light-harvesting pigment-protein complex for photosynthesis in most red algae.[1,2] Its spectral properties mainly depend on the presence of two types of covalently linked chromophores, phycourbilins (PUB) (A_{max} at 495 nm) and phycoerythrobilins (PEB) (A_{max} at 565 nm). R-PE is composed of three types of dissimilar subunits, named α, β and γ, with the formula $(\alpha\beta)_6\gamma$. The α subunit contains both PEB and PUB while the β subunit contains only PEB chromophores. The γ subunits have been partially characterized for several red algae and it was suggested that at least four chromophores were involved.[3]

In recent years, the crystal structures of hexameric B-PE, b-PE and R-PE have been determined in high resolution by X-ray crystal structure analyses,[4–6] however, the structures for the γ subunits in these proteins have never been determined, although a possible localization of the subunit was proposed. It was suggested that the γ subunit should localize in the central channel of a hexameric R-PE, therefore, it must play a very important role in the energy transfer along a PBS rod as well as within a hexameric R-PE. For this reason, separate determination of the γ subunit structure has long been attempted so as to find the structural correlation with the hexameric R-PE. In fact, up to now, there are still some problems associated with renaturation and identification of the γ subunits. Firstly, a γ subunit must have much more hydrophobic groups on the surface than α and β subunits because it localizes in the center of R-PE, which makes the isolated subunits aggregate more easily. Secondly, it is well known that isolation of protein subunits has to be carried out in highly-concentrated denaturing reagents, such as 8 M urea, and then the isolated subunits are renaturated after getting rid of the denaturing reagents through dialysis or dilution. It was found that the renaturation of the α and β subunits was relatively easy while that of the γ subunit was very difficult: this is the key problem for structural determination of the γ subunit. It is also well known that denatured proteins or polypeptides possess more stretched conformations and will become aggregated with each other by weakly non-covalent interaction in a buffer, while the denatured γ subunits must undergo even stronger self-aggregation than the α and β subunits.

Recently, a new approach, named 'artificial chaperoning', was reported for promoting the renaturation yields of denatured proteins by preventing them from aggregating.[7–11] In the first step, some kinds of detergents are needed to dissolve the aggregates by forming weakly bonded detergent–protein complexes. Secondly, the detergent molecules are stripped from the complexes by β-cyclodextrin, a drum-like molecule with an amphiphilic hollow. In this way, the detergent-free proteins will fold into their native conformation in buffer.

In the current work, the α, β and two γ subunits of R-PE from the marine red alga *Polysiphonia urceolata* were isolated and renaturated by the use of an artificial chaperone technique. The molecular weights were determined by MALDI-TOF-MS and the spectral properties were measured. It was proved that the refolding yields were several times higher than those obtained by conventional methods.

Experimental

Chemicals

All the chemicals and reagents used in this work are of analytical grade. Cetyltrimethylammonium bromide (CTAB) and β-cyclodextrin were purchased from Sigma, Bio-Gel P300, P60, P2 from Bio-Rad, and CM-Cellulose-52 from Whatman.

Preparation of R-PE

R-PE was isolated from the marine red alga *Polysiphonia urceolata* collected on QingDao beach of East China. It was purified further on a chromatography column (1.5 × 85 cm) of Bio-Gel P300 with 100–200 mesh according to the procedure described by Yu and Glazer[12] and the purity was evaluated by the absorbance ratio of A_{565}/A_{280}, which was controlled to be 5 or more in the current work.

Separation of the subunits of R-PE

All sodium phosphate buffers mentioned below contain 2 mM mercaptoethanol. For separation of the subunits, 30 mg of lyophilized R-PE were dissolved in 8 M urea acidified to pH 3.0 with acetic acid and stored for 48 hours at −10 °C in the dark.

The solution was further diluted to a concentration of 6.0 M urea (pH 3.0) and then applied to a CM-cellulose-52 column (1.5 × 20 cm) pre-equilibrated with 6 M urea (pH 3.0). The α, β and γ subunits eluted successively at the urea concentration of 6.0, 7.0 and 8.0 M respectively and were collected separately. The 8.0 M urea fraction, containing the γ subunit, was further applied to a Bio-Gel P60 (Bio-Rad) column washed with a linear concentration gradient of 0–20 mM NaCl in 8.0 M urea and the sub-fractions, γ_1 and γ_2, were collected separately.

Determination of molecular weights of the renatured subunits

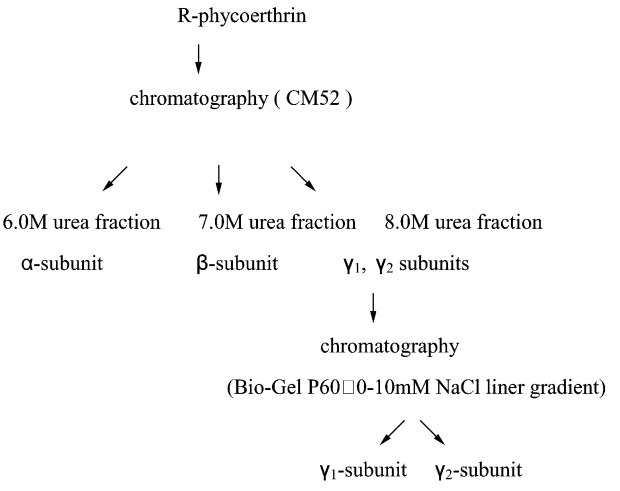
For renaturation, the 6.0–8.0 M urea solutions of the subunits were diluted with distilled water to a concentration of 3 M urea. Secondly, the diluted solutions were applied to a chromatography column of Biogel-P2 (0.8 × 15 cm) (BioRad, München) washed with distilled water. Thirdly, the separately collected samples were dialyzed against water at 4 °C to obtain the renatured subunits which were further concentrated and lyophilized for the measurement of molecular weights by MALDI-TOF-MS. The molecular weights were determined on a Biflex III MALDI-TOF mass spectometer (Bruker, Billerica, MA, USA) in Beijing MS center, Institute of Chemistry, Chinese Academy of Sciences, equipped with delayed extraction, a multi-sample probe, a TOF reflection analyzer, a nitrogen laser with wavelength 337 nm and pulse width 3 ns, and a linear flight path length of 100 cm. The flight tube was evacuated to 10^{-5} Pa and an α-cyano-4-hydroxycinnamic acid (CHCA, Acros, USA) solid matrix was used. More details for the MALDI-TOF-MS can be found in ref. 13. For MALDI-TOF-MS measurement, any salts or surfactants have to be removed from the samples, that is why distilled water, instead of buffer solution, was used in above procedures. The lyophilized subunit samples, in solid powder, should be dissolved in water before the molecular weight measurement.

Spectrophotometric measurements

Absorption spectra were obtained on a SHIMADZU UV-1601 (Japan) Ultraviolet/Visible spectrophotometer. Fluorescence excitation spectra and the quantum yield as well as the emission spectra were obtained at room temperature on a Hitachi F-4500 photometer with a 5 nm band pass, and the concentration of a sample was adjusted to have an absorption maximum of no more than 0.1. For the quantum yield measurements, Rhodamine B in ethanol was used as a standard [$\Phi = 0.94 \pm 6.5\%$].[14]

Artificial chaperone-assisted refolding

Separately collected α, β, γ_1 and γ_2 subunits, obtained as described above, were diluted with the 10 mM buffer in the presence of a detergent, resulting in the formation of a stable protein–detergent complex, then a cyclodextrin was added to the protein–detergent complex, causing the removal of the detergent from the protein, and concomitant renaturation. The detergents used for this purpose were all at around the Critical Micelle Concentrations (CMC), a species-dependent parameter.[10,15] After the diluted solution was stored for 1 hour, 20 mM β-cyclodextrin stock solution was added until the final concentrations of urea, CTAB and β-cyclodextrin reached 240, 0.9 and 5 mM, respectively. After a 3 hour incubation at room temperature, the sample was centrifuged and the supernatant was collected for spectral measurement. In the same way, Tween-80, Triton-100 and SDS, with final concentrations of 1.0, 0.3 and 8.7 mM, respectively, were also used for promoting refolding of the subunits.



Scheme 1 Separation of the subunits of R-PE in denaturing conditions.

Results

The elution sequence of the 3 kinds of subunit depends on the urea concentration, as mentioned before. The α, β and γ subunits were eluted successively with urea concentrations of 6.0, 7.0 and 8.0 M, respectively. The 8.0 M urea fraction was further separated into the sub-fractions using a Bio-Gel P60 column (1.5 × 85 cm) washed with a linear concentration gradient of 0–10 mM NaCl in 8.0 M urea (see Scheme 1).

The phycobilin number in a γ subunit has been reported to be one,[16] four [1,17,18] or even five[19] for various phycoerythrins. To our knowledge, this is the first time that two types of γ subunits of R-PE from *Polysiphonia urceolata* have been obtained. The A_{495}/A_{555} ratios in the absorption spectra of the α, γ_1 and γ_2 subunits were estimated to be 1.06, 3.22 and 5.97, respectively (see Fig. 1), which roughly reflect the relative contents of the phycourobilins (PUB) and phycoerythrobilins (PEB). Based on the published extinction coefficients, 94 000 M^{-1} cm^{-1} for PUB and 43 000 M^{-1} cm^{-1} for PEB,[12] as well as the chromophore composition of an αβ unit,[5] the chromophore contents were determined to be two PEB and one PUB for α, two PEB only for β, two PEB and three PUB for γ_1 and one PEB and three PUB for γ_2. Table 1 shows the molecular weights and the spectral properties of R-PE and the subunits.

In the dialysis method, proteins would refold as soon as the denaturant was removed. The kinetics and thermodynamics of the folding process depend on a number of factors, including

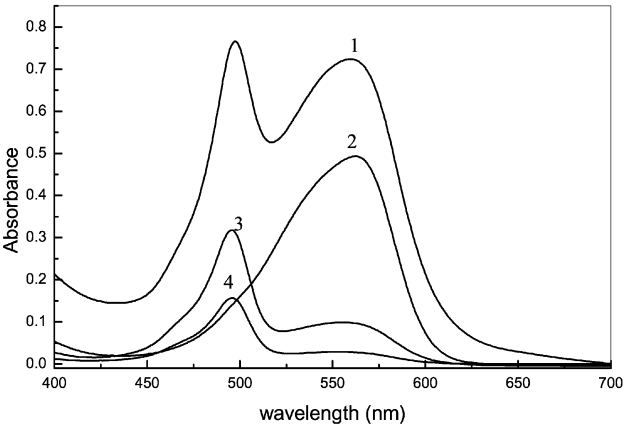


Fig. 1 Absorption spectra of denatured subunits (1 – α, 2 – β, 3 – γ_1, 4 – γ_2) in 8 M urea (pH 3.0).

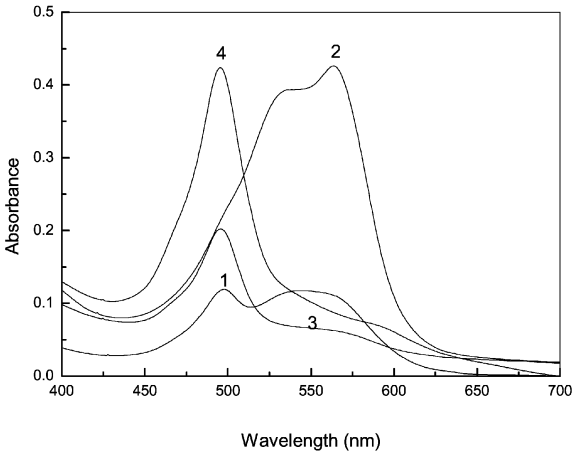


Fig. 2 Absorption spectra of the renatured subunits ($1-\alpha$, $2-\beta$, $3-\gamma_1$, $4-\gamma_2$) in 10 mM sodium phosphate buffer (pH 7.0).

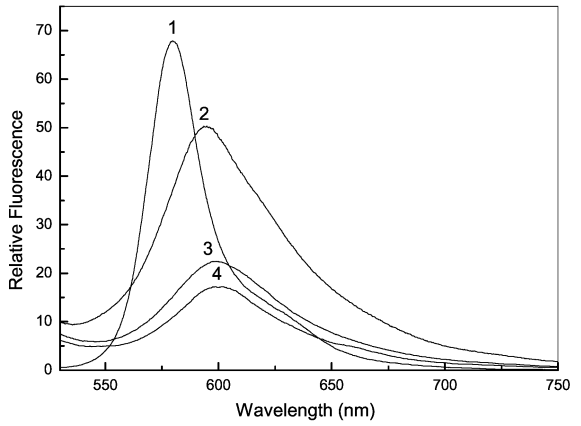


Fig. 3 Fluorescence emission spectra of the R-PE subunits renaturated by the conventional dialysis method. ($1-\alpha$, $2-\beta$, $3-\gamma_1$, $4-\gamma_2$).

topology of the amino acid sequence chain, PH, salt concentration and temperature.[20–22] It is well known that denatured proteins tend to aggregate and are not fluorescent. Refolding of the γ subunits is more difficult than that of the α or β subunit because they may contain many more hydrophobic groups, which makes the denatured γ subunits attract each other strongly and self-aggregate tightly. After the separated subunits were applied to a Biogel-P2 chromatography column and then the urea removed by dialysis, protein refolding would be taking place and result in fluorescence, which is usually taken as an indicator for renaturation of phycobiliproteins. The absorption and fluorescence spectra of the renatured subunits are shown in Fig. 2 and Fig. 3, respectively. For renaturation by the dialysis method, the concentrations of the denatured subunits must be very low (within the range 10^{-6}–10^{-7} mol L^{-1}) in order to prevent them from aggregating.

Competing with the refolding of globular proteins, the intermolecular aggregation would result in low renaturation yields. In fact, the renaturation yields for α, β, γ_1 and γ_2 subunits were only 7.6, 10.2, 2.8 and 2.2%, respectively, using the conventional dialysis method, in which the aggregated proteins were still visible.

We have also tried to renaturate the subunits by dilution with a buffer containing 2 mM DL-cystine or a combination of glutathione (GSH) and oxidized Glutathione (GSSH) in ratios of 10:1 and 5:1,[23] but the renaturation yields were even lower, 4.6, 7,2, 1.5 and 1.0% for α, β, γ_1 and γ_2 respectively.

From the data in Table 2, it can be seen that the renaturation yields of the subunits obtained by the artificial chaperoning method are much higher than those obtained by conventional methods. In this work, examining several kinds of detergent in combination with β-cyclodextrin, it was proved that the cationic detergent CTAB was optimal for renaturation of the subunits of R-PE.

Discussion

The phycobiliprotein subunits are usually separated in concentrated acidic urea in which the proteins become denatured. In fact, the γ subunits obtained through a single run on a CM-52 column were a mixture but could be separated by a further run on a Bio-Gel P60 chromatography column. The difference of the two γ subunits in the spectra can be ascribed to the chromophore compositions. It may be questioned if the two γ subunits are naturally present species or artificial products. From Table 2, it can be seen that the γ_1 subunit possesses one more chromophore but a smaller molecular weight than γ_2. It may be suggested that both of the γ subunits are naturally present rather than artificially produced for the reasons listed below. First of all, during the separation and renaturation, never had a reagent been used to break the covalent bonds so as to cut one chromophore off, in addition, γ_2 should not be a fragment of γ_1 for it possesses a larger molecular weight but less chromophores. Secondly, it has been reported for various red algae that R-PE and B-PE could have two or three γ subunits,[19] therefore, it is reasonable for R-PE to have more than one γ subunit. Thirdly, the failure to determine the entire structures of the γ subunits in R-PE, b- or B-PE by X-ray crystal structure analyses, which has been ascribed to different orientations of the γ subunit in the central channel of the R-PE,[5] may also be evidence for existence of various types of γ subunits.

It is well known that the absorption spectra of denatured phycobiliproteins are quite similar to those of the free phycobilins but less affected by the protein environment. In Fig. 1, the denatured β subunit shows a single-peak spectrum for it contains only PEB chromophores. On the other hand, in Fig. 2, the renatured β subunits show a two-peak spectrum with the maxima at 535 nm and 565 nm, which is very similar to those of the native R-PE. It can be deduced that the two PEB chromophores in a β subunit must have different protein environments.

In Fig. 3, both of the two γ subunits show single-peak fluorescence spectra with a maximum at around 600 nm, which must originate from the emission of the excited PEB chromophores. This fact implies that the γ subunits are completely renatured so that the excitation energy absorbed by the PUB could be transferred efficiently into the PEB. The peaks of

Table 1 Molecular weight and spectral properties of RPE and its subunits

	R-PE	α	β	γ_1	γ_2
Molecular weight	26 000 (6)	17 580	19 050	32 510	33 900
λ^{A}_{max}(nm) (Denatured subunit)	556.00, 495.50	556.00, 495.50	555.50	555.80, 495.80	556.20, 495.80
λ^{A}_{max}(nm) (Renatured subunit)	566.00, 536.50, 498.70	548.70, 497.40	535.60, 565.60	567.80, 500.20	568.00, 500.00
λ^{F}_{max}(nm)	577.0	579.0	596.0	600.8	601.4
Fluorescence quantum yield Φ_f	0.87 ± 0.5	0.65 ± 0.4	0.37 ± 0.5	0.25 ± 0.3	0.21 ± 0.3

Table 2 Renaturation percentages for the subunits of R-PE[a]

	α(%)	β(%)	γ_1(%)	γ_2(%)
None	7.6	10.2	12.8	2.2
CTAB, β-CD	60.4	77.9	42.5	33.9
Tween 80, β-CD	33.9	52.2	41.0	31.8
Trition-100, β-CD	39.7	72.8	37.3	30.2
SDS, β-CD	58.8	76.1	10.1	12.8

[a] The renaturation yields were determined by the use of the method described in the literature.[7,8,23]

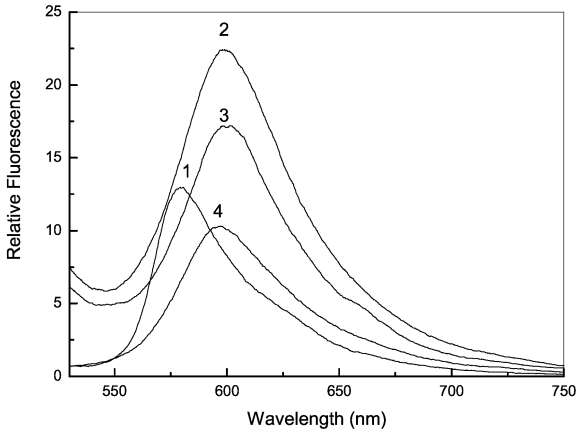


Fig. 4 Fluorescence emission spectra of the R-PE subunits renaturated by the artificial chaperone-assisted method. ($1-\alpha$, $2-\beta$, $3-\gamma_1$, $4-\gamma_2$).

the fluorescence spectra in Fig. 4 are basically the same as those in Fig. 3, which implies that the renatured subunits obtained through the two different methods are exactly the same. Further, compared with that for γ_1, the fluorescence spectrum of γ_2 is blue-shifted by several nanometers because it contains many more phycourobilins. In consideration of the compositions of the γ subunits, many more PUB chromophores greatly broaden the absorption spectrum, therefore, the light-harvesting function of R-PE is greatly strengthened. Furthermore, the γ subunits should also play a key role in excitation energy transfer in R-PE because of their localization, *i.e.*, they may be coupled with the 6 surrounding αβ units through not only the PEB but also the PUB chromophores. Therefore, determination of the structures of the γ subunits as well as the structural correlation with the hexameric $(\alpha\beta)_6$ is of key importance in elucidating the energy transfer processes along the PBS rods as well as within R-PE.

Conclusions

The artificial chaperone technique, by the use of small chemical molecules to promote protein folding, is mechanically distinct from other methods. The conventional dialysis method and the dilution additive method are usually used for renaturation of proteins, however, these methods are not so satisfactory for renaturation of the phycobiliprotein subunits, especially not for that of the γ subunits in R-PE. The application of the artificial chaperone method was proved to be successful for renaturation of the subunits of R-PE so that the γ subunits could be well characterized. Furthermore, it has long been a difficult job to determine the structure of the γ subunits for it is not easy to obtain a sufficient amount of the pure sample. Therefore, this technique also makes determination of the structures of the γ subunits possible.

Acknowledgements

The Project supported by NSFC No 30070202.

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