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R-phycoerythrins having two conformations for the same aggregate

Robert MacColl [a,b,*], Leslie E. Eisele [a]

[a] New York State Department of Health, Wadsworth Center, P.O. Box 509, Albany, NY 12201-0509, USA

[b] Department of Biomedical Sciences, University at Albany, State University of New York, Empire State Plaza, Albany, NY 12201-0509, USA

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Abstract

The visible circular dichroism (CD) spectrum of an R-phycoerythrin (*Porphyra tenera*) is composed of several positive bands. The protein in aqueous buffer very slowly exhibits changes in the CD spectrum of its chromophores, a band at 489 nm undergoes an increase in intensity and a red shift. When the band reached a 493 nm maximum, the spectrum became very stable. The aggregation state of the protein did not change during this spectral conversion. The chromophore CD spectrum was also obtained in the presence of a low concentration of urea or sodium thiocyanate, and the identical change in the CD was noted, but the change was much faster. The visible absorption and CD in the far UV spectra were unaffected by urea. Unchanged visible absorption and protein secondary structure (61% α helix) contradicted by comparatively salient alterations in the visible CD spectra suggested very subtle structural changes are influencing some of the chromophores. For a second R-phycoerythrin (*Gastroclonium coulteri*), the CD of the chromophores had a negative band on the blue edge of the spectrum. This is the first negative CD band observed for any R-phycoerythrin. Treatment of this protein with low concentrations of urea produced a change in the visible CD with the negative band being completely converted to a positive band. Fluorescence studies showed that the treatment by urea did not affect energy migration. Deconvolution of the CD spectra were used to monitor the chromophores. The results demonstrated that the same aggregate of each R-phycoerythrin could exist in two conformations, and this is a novel finding for any red algal or cyanobacterial biliprotein. The two forms of each protein would differ in tertiary structure, but retain the same secondary structures.

Keywords: Protein circular dichroism (CD); Conformation of biliproteins; Phycoerythrin; Light harvesting in photosynthesis; Conformational heterogeneity in a biliprotein

1. Introduction

Photosynthesis is initiated by the absorption of photons by an array of light-harvesting pigments. The excitons must then migrate over considerable distances to the reaction centers of photosystems I or II where the transduction of electronic to chemical energy occurs. Biliproteins are a type of photosystem II light-harvesting pigment, which are found in cyanobacteria, red algae, and cryptomonads. They can be isolated as discrete particles containing various numbers and types of open-chain tetrapyrrole chromophores. The variety of chromophores pro-

* Corresponding author. Wadsworth Center, NYSDOH, P.O. Box 509, Albany, N.Y. 12201-0509, e-mail: robert.maccoll@wadsworth.org; Tel.: (518) 474-3728, Fax.: (518) 474-7992.

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duces a spectrally diverse group of proteins, the main types being allophycocyanins, phycocyanins, phycoerythrocyanin, and phycoerythrins. Reviews of biliproteins are available [1–6].

R-Phycoerythrin is among the most complex spectroscopic types of biliproteins. The protein is isolated from red algae with a protein structure of $\alpha_6\beta_6\gamma$. The chromophore contents of the α and β polypeptides are two phycoerythrobilins, and two phycoerythrobilins and one phycourobilin, respectively [7]. The γ polypeptide has a variable chromophore content, and three of the reported results are: one phycoerythrobilin and three phycourobilins, three phycourobilins and two phycoerythrobilins, and four phycourobilins and one phycoerythrobilin [7–9]. The chromophores of R-phycoerythrin have also been studied by complexing them to zinc ions [10]. Energy migration among these chromophores is usually assumed to occur by a resonance mechanism between dipole pairs [11,12]. More than one γ polypeptide of R-phycoerythrin is found in each red alga [13–16]. For *Callithamnion corymbosun*, three γ polypeptides were detected, each having the same chromophore distribution, and for *Antithamnion sparsum* two γ polypeptides with different chromophore contents were found [13].

The spectrum resulting from these chromophores is a complex of overlapping bands, which are studied in this work by absorption, circular dichroism (CD), fluorescence spectroscopy and spectroscopic deconvolutions. In particular, there have been very few CD studies on the R-phycoerythrins, and this technique will be used to obtain insights into their chromophore organization. The two red algae that were the sources of the R-phycoerythrins were *Gastroclonium coulteri* and *Porphyra tenera*.

2. Materials and methods

R-Phycoerythrin from *G. coulteri* was obtained as an ammonium sulfate (60% saturated) precipitate (Schweizerhall, Inc.). The precipitated protein was centrifuged and the supernatant discarded. The pellet was dissolved in pH 6.0, 0.1 ionic strength, sodium phosphate buffer and dialyzed into this buffer to remove residual ammonium sulfate. After completion of dialysis, the protein solution was briefly centrifuged to remove insoluble protein. It was shown to be pure by a A_{565}/A_{280} ratio of 4.7 [17]. Further proof of purity was determined by gel-filtration column chromatography. The gel-filtration experiments were performed on a Waters high-performance liquid chromatography system; a photodiode array detector allowed the determination of the spectrum of any material eluting from the column. It was determined that R-phycoerythrin was very pure since it was the only protein detected. The purified protein was stored cold in 60% saturated ammonium sulfate.

The second R-phycoerythrin was from *P. tenera* (Pierce). It was treated similarly to the first protein, and was likewise found to be very pure. It was stored under 60% saturated ammonium sulfate in the cold. When needed for study, purified protein was dialyzed into pH 6.0 buffer.

Absorption spectra were obtained on a DU640 spectrophotometer (Beckman). The temperature of the samples was maintained at 23°C using a Peltier device. Protein concentration was based on an absorptivity of 8.2 for a 1 mg ml^{-1} solution at 565 nm [15].

CD spectra were recorded on a JASCO J-720 spectropolarimeter. Each spectrum was obtained a number of times and the average spectrum recorded to improve the signal-to-noise ratio. A solvent spectrum was recorded in the same manner and subtracted from each sample spectrum. The spectra were then smoothed using a Savitzky-Golay filter. The temperature was maintained at 23° by water circulating from a Neslab refrigerated circulator, RTE-111. A 1 cm light path was used for CD in the visible region and absorption measurements. CD in the far UV was performed using a 0.5 mm light path. CD data was converted to molar units for calculation of secondary structures. Protein concentrations were 0.093 and 0.068 g L^{-1}.

Fluorescence emission spectra were obtained using a Perkin–Elmer model LS 50B fluorescence spectrophotometer. Temperature was kept at 23° by circulating water from a Neslab refrigerated circulator, RTE-111. Solutions for fluorescence were at $A_{565} = 0.05$ or 0.10 in a 1 cm light path in order to minimize the reabsorption of emission. Protein concentration was about 0.0066 or 0.013 g L^{-1}. Fluorescence (excitation) polarization spectra were obtained

with the emission at 600 nm. The polarization was calculated by:

$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where G was an instrument correction factor, I_{VV} was the intensity when the sample was excited with vertically polarized light and the emission was monitored with a vertical polarizer, and I_{VH} was the intensity when the sample was excited with vertically polarized light and emission was monitored with a horizontal polarizer.

Samples of R-phycoerythrin in urea were prepared by mixing a pH 6.0 solution of 8.0 M urea with the protein solution. A non-urea sample was prepared in the same way but with mixing of the sample with neat pH 6.0 buffer. Samples were let stand in a refrigerator overnight before measurements were performed to ensure a completed urea effect. The 8.0 M urea was prepared by dissolving urea in the pH 6.0 buffer and titrating with dilute acid back to pH 6.0. A Hanna pH meter was used. Solutions of NaSCN were handled in a similar manner using a 2.0 M NaSCN stock solution in pH 6.0 buffer.

Spectroscopic deconvolution was performed on the CD spectra using the Jandel PeakFit software. The method was interactive using a Levenberg-Marquardt fitting. Four types of bands were tried: Gaussian, Lorentzian, Voight, and Pearson VII. These bands were applied to the spectra either alone or in combinations. For the R-phycoerythrins, the number of components was chosen based on discernible features in the CD spectra.

Gel-filtration column chromatography to obtain molecular weights was employed using a Waters 625 liquid chromatography system equipped with a 717 autosampler and a 996 photodiode array detector. The column was a Waters Protein Pak 300 SW column with 8.0 × 300 mm dimensions. The flow rate was 0.8 mL per min. The column was operated with isocratic conditions using pH 6.0 buffer. The molecular weight calibration of the column was obtained from running the following standards: vitamin B12, 1,350; myoglobin, 17,000; ovalbumin, 44,000; gamma globulin, 158,000; and thyroglobulin, 670,000 molecular weight (Bio-Rad).

3. Results and discussion

3.1. R-Phycoerythrin (P. tenera)

R-Phycoerythrin was obtained from the red alga, *P. tenera*. Gel-filtration column chromatography in pH 6.0 buffer showed the solution to essentially contain a single protein at about 220,000 molecular weight (data not shown). This size corresponds to the $\alpha_6\beta_6\gamma$ polypeptide structure, which characterizes the R-phycoerythrins. The absorption spectrum is also typical of R-phycoerythrin (Fig. 1). The CD spectrum was complex having a very small positive band at 489 nm, a shoulder at about 510 nm, and maxima at 535 and 567 nm (Fig. 2).

Protein, for example, if left for a long time in solution, is also obtained having the visible CD spectrum changed with the very small positive band increasing in intensity and shifting toward longer wavelengths (data not shown). Gel-filtration column chromatography of this changed R-phycoerythrin shows no sign of dissociation and the identical molecular weight, about 220,000, found for the protein with 489 nm CD band (data not shown). This second type, once a maximum at 493 nm is obtained, is very stable. Therefore, there are two forms of the $\alpha_6\beta_6\gamma$ protein having different conformations: one type having the very small band at 489 nm and a second type having the more intense 493 nm band. This is the first time any cyanobacterial or red algal biliprotein has been shown to exist as a single aggregate with two different conformations. Perhaps X-ray crystallography of these two spectral forms would determine the basis of the difference.

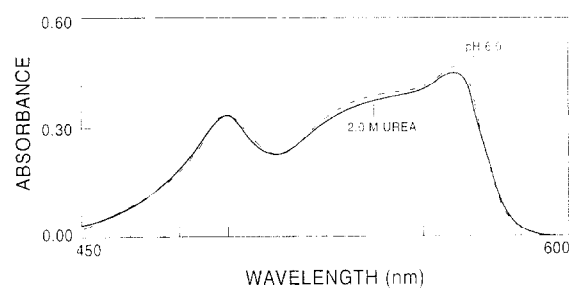

Fig. 1. Absorption spectra of R-phycoerythrin (*P. tenera*) in pH 6.0 buffer and pH 6.0 buffer plus 2.0 M urea. Temperature was 23°C. Light paths were 1 cm.

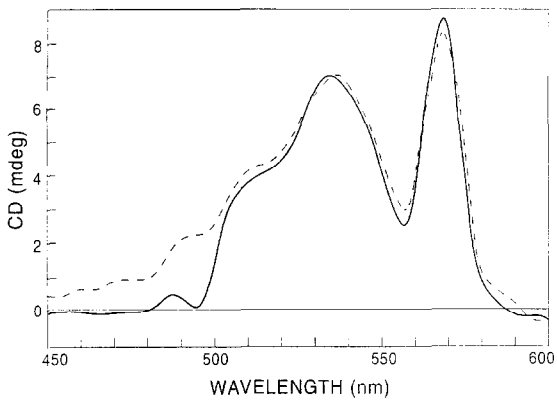

Fig. 2. The CD spectra of R-phycoerythrin, (*P. tenera*). Spectra were taken at 23°C in pH 6.0 buffer (solid line) and in pH 6.0 plus 2.0 M urea (dashed line). The pH 6.0 spectrum was in a 0.5 cm light path at an $A_{565} = 1.0$, and the 2.0 M urea spectrum was in a 1 cm light path at an $A_{565} = 0.5$. Solutions of R-phycoerythrin at identical protein concentrations and light paths yielded similar results.

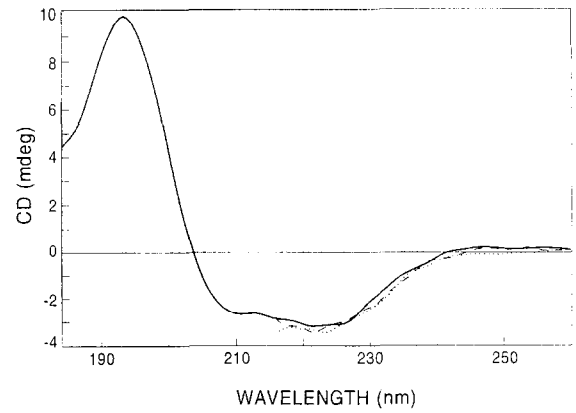

Fig. 3. CD spectra in the far UV of R-phycoerythrin. Spectra are shown for protein in pH 6.0, buffer (solid line) and that buffer plus either 0.8 M (dashed line), or 2.0 M urea (dotted line). The protein concentration was the same for the three samples. Source of protein was *P. tenera*.

Can the transformation between the two forms of R-phycoerythrin be accelerated? A solution of R-phycoerythrin was made 2.0 M urea and the visible CD spectrum obtained (Fig. 2). There was clear evidence of a small CD increase at the blue edge of the spectrum. The small blue-edge positive band also red shifted to about 493 nm yielding the identical CD spectrum as found for some samples not treated by urea. Unlike the CD spectra, the visible absorption spectra were identical in pH 6.0 or pH 6.0 plus urea (Fig. 1). This absorption result demonstrated that 2.0 M urea did not denature the protein. The same process was tried with 1.0 M NaSCN and again a similar CD change at the blue edge was obtained (data not shown). NaSCN is a chaotrope that has been used to study other biliproteins [3].

The CD spectra in the far UV gives information on the secondary structure of proteins. The CD spectrum of R-phycoerythrin in pH 6.0 buffer has a positive maximum at 193 nm and negative maxima at 211 and 220 nm (Fig. 3). This spectrum indicates a high α-helix content and calculations confirmed this finding [18]. The estimate yielded a secondary structure of 61% α helix, 13% β sheet, 14% turn, and 12% other. Urea cuts-off access to much of the far UV, but the spectra in 0.8 and 2.0 M urea show no signs of any change in secondary structure produced by these low concentrations of urea. The visible absorption spectrum was not affected by 2.0 M urea (Fig. 1). These observations indicated that the overall effects of 2.0 M urea on the protein and its chromophores were small. They suggest that the two forms of R-phycoerythrin (Fig. 2) are produced by a change in tertiary structure.

To try to learn more about the chromophores of this R-phycoerythrin, urea was added to a pH 6.0 protein solution to yield a series of concentrations of urea (Fig. 4) at a constant protein concentration. The CD spectra in 0.8 and 2.0 M urea are clearly much like the native protein (Figs. 2 and 4). The results

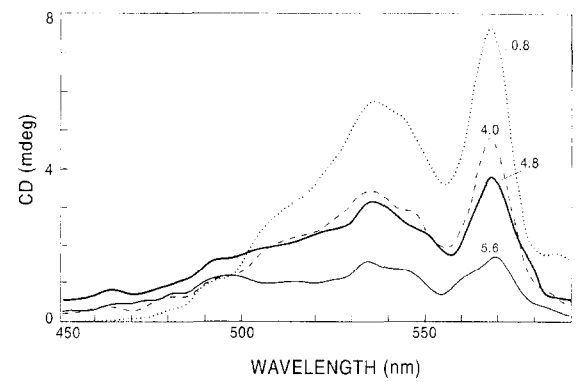

Fig. 4. Effects of urea on the CD of R-phycoerythrin, (*P. tenera*). Spectra were taken at 23°C in a 1 cm light path. Protein concentrations were identical for all solutions. Numbers on the curves refer to molar concentrations of urea.

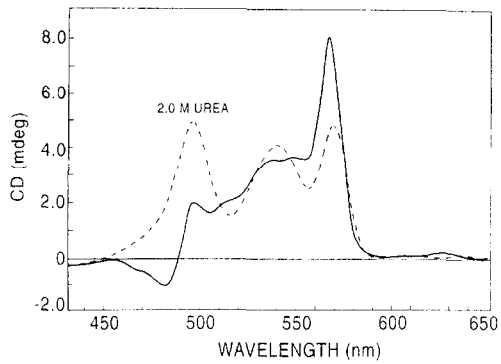

Fig. 5. Visible CD spectra of R-phycoerythrin, (*G. coulteri*), in pH 6.0 buffer and pH 6.0 buffer with 2.0 M urea. Temperature was 23°C. The path length was 1 cm. Protein concentration was 0.068 g L^{-1} for both samples.

show a lower intensity is produced by urea throughout most of the spectrum. In general, any substance that changes the conformation of any biliprotein usually lowers the visible intensity because chromophores tend to become less linear and thus lower in absorption and CD in the first excited state. Therefore, the increase in CD with 2 M urea on the blue edge (Fig. 2) is very unusual. A suggestion that the increase may be produced by elimination of a hidden negative is therefore reasonable. The various results with 2 M urea are stable at least during the experimental day.

3.2. R-Phycoerythrin (G. coulteri)

The absorption spectrum of R-phycoerythrin isolated from the red alga, *G. coulteri*, shows visible maxima at 494 and 565 and a shoulder at 545 nm. The visible CD spectrum of R-phycoerythrin (*G. coulteri*) at pH 6.0 is very complex, and a negative band is observed at the blue edge of the spectrum (Fig. 5). Previous CD studies reported only positive bands. A CD spectrum of R-phycoerythrin isolated from *Ceramium rubrum* did not show the negative band [19]. Likewise, a CD spectrum of R-phycoerythrin isolated from *Gracilaria longa* was described as having three positive bands at 497, 535 and 568 nm [17]. The novel negative CD band is located at 481 nm, and this is the region where phycourobilin absorption predominates, indicating that this chromophore may produce the transition. The shapes of the positive bands also varied between the *C. rubrum* and *G. coulteri* spectra, and it is possible that R-phycoerythrins isolated from various algae will have different CD spectra.

Could a spectroscopic change similar to that induced in R-phycoerythrin from *P. tenera* also occur for this protein? When the 2.0 M urea is added to the R-phycoerythrin, the CD spectrum shows a dramatic change in appearance (Fig. 5). The most noteworthy aspect is the disappearance of the negative band. The positive bands increase or decrease in rotational strength, and the bands had maxima at 496, 538 and 567 nm. The hidden bands at 514 and 551 nm (Fig. 5) were eliminated. The visible absorption spectrum, however, was little changed (data not shown), as previously shown for the *P. tenera* protein (Fig. 1).

What causes the spectral changes when the R-phycoerythrin is treated with 2.0 M urea? Gel-filtration column chromatography monitored at 565 nm showed that the R-phycoerythrin at pH 6.0 was composed of a major band of over 200,000 molecular weight. The solution had a very minor band at a larger molecular weight (Fig. 6). The spectrum of both the bands, obtained from the photodiode array

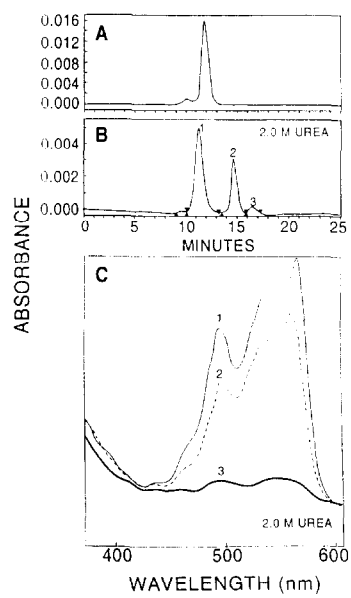

Fig. 6. Gel-filtration chromatography of R-phycoerythrin (*G. coulteri*), in pH 6.0 buffer and pH 6.0 with 2.0 M urea. The detection wavelength was at 565 nm in A and B. Spectra were taken using the photodiode array detector system. The numbers on the bands in B correspond to the numbers in C.

detector, corresponds to that of R-phycoerythrin (data not shown). The main band is assigned to the expected $\alpha_6\beta_6\gamma$ polypeptide structure and the very minor band is probably a dimer, $(\alpha_6\beta_6\gamma)_2$.

The influence of 2.0 M urea at pH 6.0 is to produce two additional lower-molecular-weight bands at about 44,000 and 24,000 molecular weight. The band corresponding to $\alpha_6\beta_6\gamma$ represents over 60% of the total 565 nm absorbance, indicating that the rest of the protein had dissociated. The dissociation band at about 44,000 molecular weight has a spectrum showing less 490 nm absorption than intact $\alpha_6\beta_6\gamma$ R-phycoerythrin (Fig. 6C). The ratio of A_{565}/A_{490} for the 200,000, 44,000, and 24,000 molecular-weight bands were 1.47, 1.68, and about 1, respectively. The 44,000 molecular weight and the spectrum are both indicative of the $\alpha\beta$ polypeptide unit of the protein since the molecular weights of both the α and β polypeptides are approximately 20,000 and the γ polypeptide is highly enriched in phycourobilin (490 nm maximum). The 24,000 molecular weight band is probably individual polypeptides, which have expected molecular weights of 20,000 to 33,000. The effect of 2.0 M urea, for 40% of the protein, would probably be: $\alpha_6\beta_6\gamma \rightarrow 6\alpha\beta + \gamma$. Some of the $\alpha\beta$ monomers could continue to dissociate to individual α and β polypeptides. The CD results suggest that the $\alpha_6\beta_6\gamma$ aggregate must have undergone a conformational change in 2 M urea, as well as partially dissociating.

The fluorescence emission spectra of R-phycoerythrin (*G. coulteri*) can give information on energy transfer, and were obtained in pH 6.0 buffer and 2.0 M urea, pH 6.0. The samples were excited at 470 nm and the emission spectrum in 2.0 M urea was similar in shape and maximum (575 nm). Exciting the protein at 470 nm will affect only the highest-energy chromophores, and emission with a 575 nm maximum can occur only after the energy migrates to the lowest-energy chromophores.

Deconvolutions of the CD spectra of R-phycoerythrin (*G. coulteri*) were carried out in order to obtain data on the specific chromophores, or energetically-similar groups of chromophores (Fig. 7). The deconvolution was carried out using six components: the five positive bands were Gaussian and the negative band was Lorentzian. The choice of six components was based on visual observations of some

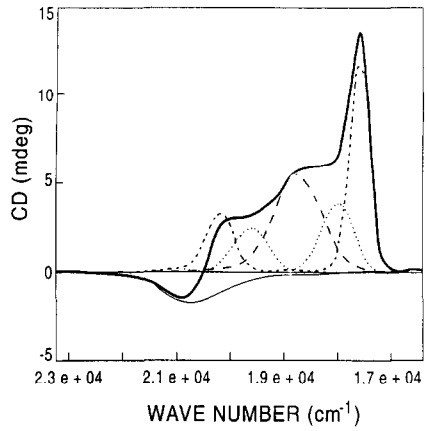

Fig. 7. Deconvolution of the CD spectra of R-phycoerythrin, (*G. coulteri*). R-phycoerythrin was in pH 6.0 buffer. The heavy lines are the experimental data, and the light, dotted, or dashed lines are the fitted components. The heavy line of the experimental data totally covers a line for the sum of the components, and this suggests a good fit of the data.

subtle features in the CD spectrum (Fig. 5). The maxima were 567, 554, 533, 509, 495, and 484 nm for a pH 6.0 solution of the protein. For the 2.0 M urea sample, the CD spectrum was best fitted by four Gaussian components at 568, 539, 500, and 489 nm. Other deconvolutions are possible but these serve to highlight the main features of the component spectra. The selected deconvolutions had the best statistical fits of many different attempts. The fewer components required to fit the 2.0 M urea spectrum demonstrated that the chromophores are organized into fewer energy states.

4. Conclusion

Why was 2.0 M urea selected for this study, since urea in high concentrations is a potent protein denaturant? Timasheff [20] has noted that, in general, the unfolding and denaturing effect on proteins began at 3.0 M urea, and the thermodynamic basis for this effect was discussed. Therefore, 2.0 M urea is a concentration at which the effect of urea will be more gentle and denaturation (unfolding) should not occur. For R-phycoerythrin (*P. tenera*), this point is demonstrated by the visible absorption spectrum (Fig. 1), which shows the large changes signaling the denaturation of a biliprotein are totally absent, and

by the fact that the secondary protein structure is unchanged (Fig. 3). At higher urea concentrations, the spectroscopic changes caused by denaturation are apparent (Fig. 4).

Most important for an appreciation of what is occurring is that, for the protein from *P. tenera*, the CD change can occur without urea treatment and without any protein disaggregation. This two tier organization of the chromophores of R-phycoerythrin is the first time such a variable topography on the same aggregate size have been observed from any cyanobacterial or red algal biliprotein. This suggests that the assembly to the $\alpha_6\beta_6\gamma$ structure may arrive at an intermediate tertiary conformation of $\alpha_6\beta_6\gamma$. This intermediate form then proceeds to the final form. The rearrangement effects the chromophores, which may attain better positioning and chromophore-chromophore interactions necessary for highly efficient energy migration. This final conformation may be obtained upon assembly of $\alpha_6\beta_6\gamma$ onto the phycobilisome.

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