BBA 45514

MERCURIAL-INDUCED DISSOCIATION OF PHYCOERYTHRIN FROM *CERAMIUM RUBRUM*

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(Received July 1st, 1966)

SUMMARY

1. The effect of the mercurial, *p*-chloromercuribenzoate (PCMB), upon phycoerythrin isolated from *Ceramium rubrum* has been studied.

2. Gel filtration permitted the separation of 4 types of subunit from the mercurial-treated phycoerythrin. The first, an insoluble protein, exhibited a major absorption maximum at about 500 mμ and a subsidiary band at about 550 mμ. The second, a soluble protein, exhibited 2 absorption maxima at 500 and 545 mμ. The third and fourth are also soluble and absorb at 493 and 495 mμ, respectively. The fourth protein is thought to be a soluble form of the insoluble protein since it also exhibits an absorption band around 550 mμ.

3. Removal of the mercurial from the unfractionated subunits with glutathione did not bring about complete reconstitution of the native phycoerythrin. However, the second subunit was found to undergo considerable reassociation.

4. The reassociated form of the second subunit exhibited a high degree of regeneration of the 565 mμ absorption band suggesting a specific relationship between the 565 mμ chromophore and protein conformation.

INTRODUCTION

Recent investigations[1] have shown that phycoerythrin obtained from the red alga *Porphyridium cruentum* consists of 2 kinds of subunit, each containing a different pigment moiety. Phycoerythrobilin which is probably contained in one of the subunits is responsible for absorption bands at 545 and 565 mμ. The absorption at 500 mμ can be attributed to phycourobilin present in the other subunit. This phycoerythrin is characterized by its major absorption maximum at 545 mμ, a subsidiary band at 565 mμ, and a shoulder at 500 mμ.

Phycoerythrin from the red alga *Ceramium rubrum* differs from *P. cruentum* phycoerythrin in that it exhibits 3 distinctly separated absorption bands in the visible region at 495, 540, and 565 mμ. Previous studies[2] have shown that *p*-chloromercuribenzoate (PCMB) effects a spectral change of this phycoerythrin, most prominently manifested by the elimination of the 565 mμ band. A similar phenomenon is found to occur with phycoerythrin from *P. cruentum*[1].

Abbreviation: PCMB, *p*-chloromercuribenzoate, sodium salt.

This paper presents the results of our study of the PCMB-induced dissociation of phycoerythrin from *C. rubrum*. These dissociated subunits were separated and their spectral properties were characterized. Reassociation of certain subunits related to the regeneration of the 565 mμ band will be shown to occur upon removal of the PCMB with the sulfhydryl compound, glutathione.

MATERIALS

Chemicals were obtained commercially: in particular, glutathione (reduced) and PCMB, from California Corporation for Biochemical Research (Los Angeles, Calif.); Sephadex G-100 from Pharmacia Fine Chemicals, Inc. (New Market, N.J.).

The red marine alga *C. rubrum* was collected at the seashore near Woods Hole, Mass., frozen, and stored in a deepfreeze until ready for use.

METHODS

To prepare phycoerythrin, frozen portions of *C. rubrum* were allowed to defrost at room temperature. Enough 0.1 M phosphate buffer (pH 7.0) was added to cover the thawed mass. The resulting mixture was homogenized for 3 min using a Waring blendor. The homogenate was allowed to stand for several days at 4° and then strained through gauze. The strained material was centrifuged at 13000 × *g* for 30 min. The strongly colored supernatant fluid was mixed intimately with an equal volume of 1-butanol (ref. 3) and centrifuged at 10000 × *g* for 30 min. The lower aqueous phase was removed and made 30 % satd. with $(NH_4)_2SO_4$. The mixture was then centrifuged at 30000 × *g* for 30 min. The crude phycoerythrin was removed, suspended in 0.1 M phosphate buffer (pH 7.0) and dialyzed overnight against 2 changes of the same buffer. The diffusate was centrifuged at 10000 × *g* for 15 min to remove the bulk of insoluble material. The supernatant fluid was then recentrifuged at 100000 × *g* for 60 min to remove remaining traces of insoluble material. Only a single precipitation with 30 % $(NH_4)_2SO_4$ was found to be necessary to obtain a product whose ratio of absorbance at 545 mμ to that at 275 mμ was greater than 4.0. Highly concentrated solutions of phycoerythrin were obtained as reported previously[1].

Gel filtration studies, sedimentation analyses, and protein concentration determinations were carried out as described previously[1].

RESULTS

Fig. 1 shows how the absorption spectrum of phycoerythrin in 0.1 M phosphate buffer at pH 7.0 changes when the PCMB-saturated mixture was allowed to stand for a week at 4° and how the subsequent addition of glutathione effects a partial reconversion of the PCMB-induced spectral change to the original state. Saturating the phycoerythrin with PCMB not only completely eliminated the 565 mμ absorption maximum but it also decreased the intensity of the 540 and 495 mμ bands accompanied by a shift of the bands to slightly longer wavelengths. An increase in absorption at about 600 mμ was also observed. Upon the addition of glutathione, an incomplete but definite regeneration of the 565 mμ band occurred together with a further decrease in absorption of the 500 mμ band shifting back to its original wavelength.

Since phycoerythrin from *P. cruentum* yielded an insoluble purple subunit when treated with PCMB, which was easily separated by centrifugation[1], we decided to employ the same procedure on the above PCMB-treated phycoerythrin from *C. rubrum*. After centrifuging at 198000 × *g* for 2 h, we were able to isolate an insoluble purple component. A red component remained in the supernatant fluid. The absorption spectrum of the insoluble purple subunit, which had been washed several times with 0.1 M phosphate buffer (pH 7.0) and resuspended in the same buffer, is shown in Fig. 2. The major absorption peak is at about 500 mμ with a secondary band around 550 mμ extending beyond 600 mμ. Light scattering effects superimposed upon real absorption are indicative of particulate matter in suspension. The supernatant fluid containing the red subunit exhibited 2 distinct maxima at 500 and 545 mμ with a slight shoulder at about 595 mμ (Fig. 2).

The sedimentation patterns of the untreated and PCMB-treated phycoerythrin are shown in Fig. 3. Native phycoerythrin was found to have an $s^0_{20,w}$ value of 11.6 S, which is in good agreement with the value of 11.9 S reported by VAUGHAN[4]. This is comparable to the value of 11.5 S for phycoerythrin from *P. cruentum*[1]. The upper pattern represents phycoerythrin treated with excess PCMB and allowed to stand for a week at 4°. The resulting mixture was filtered through glass wool to remove undissolved PCMB prior to ultracentrifugation studies. The insoluble purple component, which is barely perceptible in Fig. 3, moved much faster than the slow-moving component corresponding to the soluble red subunit. The $s_{20,w}$ value of this red subunit, as obtained above, was found to be 3.3 S. After addition of glutathione to

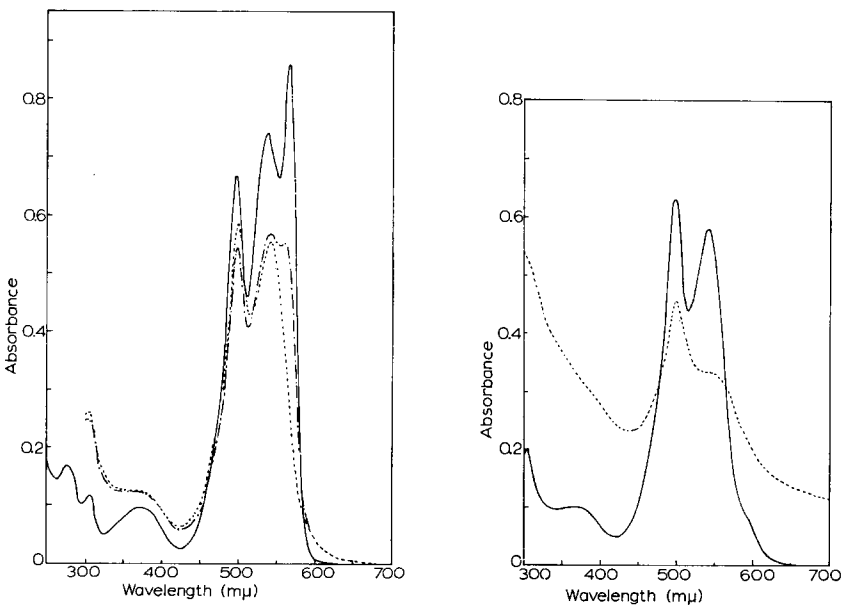


Fig. 1. Absorption spectra of diluted solutions of phycoerythrin (———), phycoerythrin saturated with PCMB (– – – –), and phycoerythrin saturated with PCMB, then treated with $1.5 \cdot 10^{-2}$ M glutathione (–•–•–) in 0.1 M phosphate buffer (pH 7.0).

Fig. 2. Absorption spectra of the purple precipitate (– – – –) and red supernatant fluid (———) separated by centrifugation, in 0.1 M phosphate buffer (pH 7.0).

the PCMB-treated phycoerythrin, the $s_{20,w}$ value became 4.0 S (Fig. 4), indicating that reconstitution to native phycoerythrin was incomplete. As shown in Fig. 4, after 84 min of centrifuging at 50740 rev./min, a marked broadening of the slow-moving peak is seen. Since such a broadening was not evidenced in the absence of glutathione after 87 min of centrifuging at the same speed (Fig. 3), its presence is suggestive of a heterogeneous mixture containing associated forms of the red subunit.

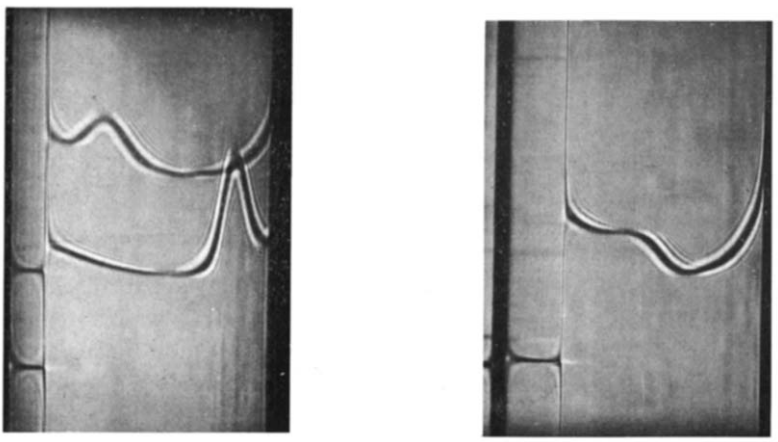


Fig. 3. Sedimentation pattern of native phycoerythrin (about 0.8%, lower pattern) and phycocrythrin (about 0.8%) saturated with PCMB and filtered through glass wool (upper pattern). Solvent: 1% NaCl–0.1 M phosphate buffer (pH 7.0). Photographs were taken 87 min after the centrifuge reached 50740 rev./min. Menisci are on the left. Temperature, 20°; phaseplate angle, 60°.

Fig. 4. Sedimentation pattern of phycoerythrin (about 0.8%) saturated with PCMB, filtered through glass wool and then treated with $1.5 \cdot 10^{-2}$ M glutathione. Solvent: 1% NaCl–0.1 M phosphate buffer (pH 7.0). Photograph was taken 84 min after the centrifuge reached 50740 rev. per min. Meniscus is on the left. Temperature, 20°; phaseplate angle, 60°.

The above results show that phycoerythrin from *C. rubrum* is split into at least 2 subunits, one purple and the other red. The red subunit tends to reassociate partially upon the addition of glutathione. Gel filtration studies not only confirm these findings but also uncover the presence of 2 other separate components heretofore unreported. Fig. 5 shows the results of the Sephadex G-100 gel filtration experiments. Native phycoerythrin moved on the column as a single band. With the PCMB-treated phycoerythrin, 4 bands appeared, purple, lowermost, followed by red, brown and purple in that order on the column. Absorption spectra of selected fractions from these bands are shown in Fig. 6. Representative Fractions 12 and 13 of the purple band moving fastest on the column exhibited absorption spectra, including light scattering effects, identical to that of the insoluble purple suspension separated by centrifugation (see Fig. 2). The spectrum of Fraction 20 from the following red band was almost identical to that of the red supernatant fluid separated by centrifugation except for the diminished shoulder at 595 mμ. Spectra of Fractions 32 and 37 from the slowly moving brown and purple bands exhibited major maxima at 493 and 495 mμ, respectively. Differences in their spectra occur between 500 and 600 mμ. Fraction 37 from the purple band exhibited a higher absorption in this range and

even beyond. Since this soluble purple component is present in the supernatant fluid containing the red subunit, it may account for the shoulder observed at about 595 mμ in the spectrum of the red supernatant fluid (see Fig. 2). When excited with 366 mμ ultraviolet light, the brown component exhibits a greenish fluorescence and the purple component a reddish fluorescence. These 2 components, because of their presence in relatively small amounts and their low molecular weights, did not appear in the ultracentrifugal pattern of Fig. 3.

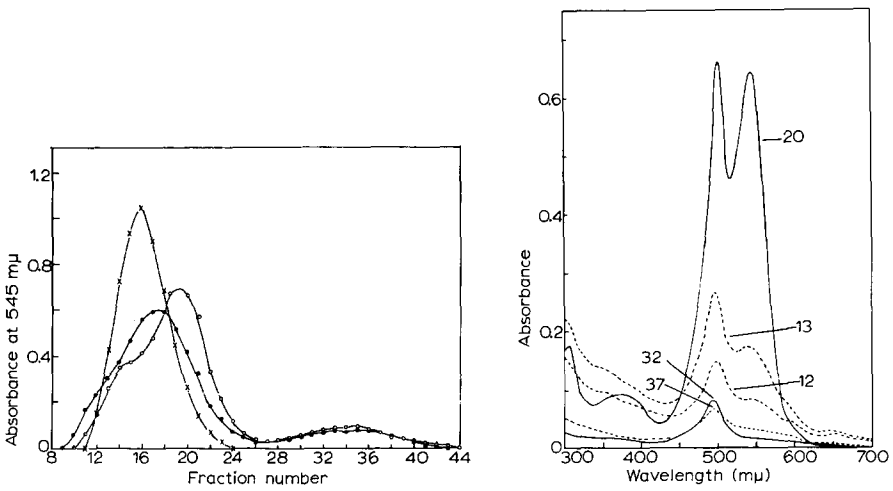


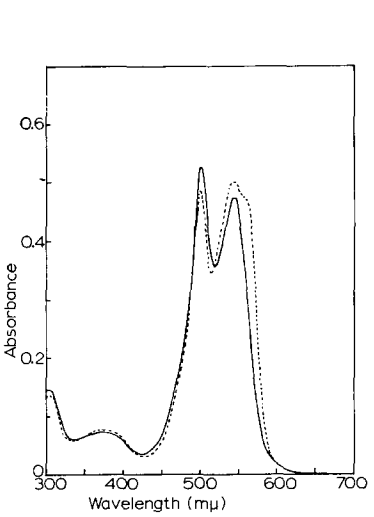
Fig. 5. Sephadex G-100 gel filtration of phycoerythrin (×), phycoerythrin saturated with PCMB (○), and phycoerythrin saturated with PCMB, then treated with $1.5 \cdot 10^{-2}$ M glutathione (●) in 0.1 M phosphate buffer (pH 7.0).

Fig. 6. Absorption spectra of Fractions No. 12, 13, 20, 32, and 37 from the gel filtration of phycoerythrin saturated with PCMB.

Subsequent treatment of the fractions from both purple bands and the brown band with glutathione effected no changes in their spectral characteristics. Fractions from the red band did, however, undergo changes in their spectra when treated with glutathione. This change (see Fig. 7) was almost identical to the spectral change occurring with the PCMB-treated phycoerythrin upon treatment with glutathione (Fig. 1). It is obvious from Fig. 7 that the removal of PCMB from the red subunit by glutathione partially regenerates the 565 mμ chromophore. This indicates that the 565 mμ chromophore had been present in the red subunit but in the course of PCMB treatment it was modified in such a manner so that its absorption was effectively masked by the other 2 bands.

It is shown in Fig. 8 that this regeneration of the 565 mμ chromophore is closely related to the reassociation of the more abundant red subunit. The results of the gel filtration studies (Fig. 5) show that the red band of the PCMB-treated phycoerythrin to which glutathione had been added before gel filtration, moved faster than that of the PCMB-treated phycoerythrin without glutathione. It is apparent that the other 3 bands were little changed. This provides convincing evidence that the red subunit may have undergone considerable reassociation upon the addition of glutathione giving rise to heavier species which move faster on the column.

This premise has already been suggested as an explanation for the broad peak observed in the sedimentation pattern of Fig. 4. The absorption spectra of selected fractions from the gel filtration of the PCMB-treated phycoerythrin containing glutathione are shown in Fig. 8. As can be seen, the leading Fractions 13 and 15, of the red band reveal a better recovery of the 565 mμ band than does the trailing fraction (Fraction 21). The middle fraction, (Fraction 17), shows an intermediate recovery.



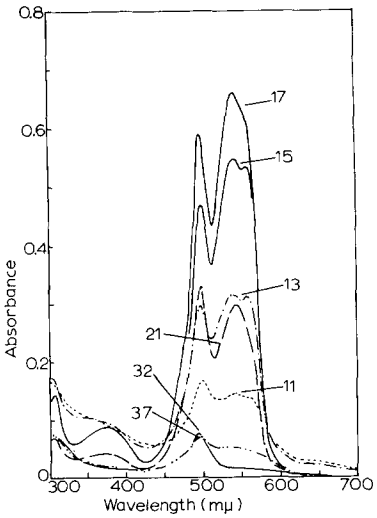


Fig. 7. Absorption spectra of Fraction 21 from the red band from the gel filtration of phycoerythrin saturated with PCMB, in the absence of glutathione (———) and in the presence of 10^{-3} M glutathione (– – – –).

Fig. 8. Absorption spectra of Fractions No. 11, 13, 15, 17, 21, 32, 37 from the gel filtration of phycoerythrin saturated with PCMB, then treated with $1.5 \cdot 10^{-2}$ M glutathione.

These results indicate that the associated red subunit contains a larger amount of the regenerated 565 mμ chromophore than does the less associated or unassociated red subunit.

DISCUSSION

Phycoerythrin from *C. rubrum* is split into 4 kinds of subunit when treated with PCMB. One is an insoluble purple subunit exhibiting a major absorption maximum at about 500 mμ. It resembles the insoluble purple subunit obtained from PCMB-treated *P. cruentum* phycoerythin perhaps with the exception of small differences in their degree of solubility. Also present is a soluble purple component which appears to exhibit similar spectral characteristics as the insoluble purple component. This suggests that these 2 purple components may indeed be a form of the same subunit, one soluble in 0.1 M phosphate buffer (pH 7.0), the other not. On the other hand, no evidence for the dissolution of the purple subunit from *P. cruentum* phycoerythrin was observed under identical conditions. Since the soluble form of the purple subunit is the smallest, the other purple component apparently is an insoluble aggregate of these smaller purple subunits. This heavy aggregate moves faster than the red subunit

either during ultracentrifugation or gel filtration. The brown subunit, intermediate in size between the soluble purple subunit and the red subunit, is present in relatively small amounts and is characteristic of phycoerythrin from *C. rubrum*. It has not been found in phycoerythrin from *P. cruentum*.

The spectrum of the highly abundant red subunit exhibits 2 absorption maxima at 545 and 500 mμ and differs from the single-peaked (545 mμ) red subunit of *P. cruentum* phycoerythrin in this respect. However, the sedimentation coefficients of each of these red subunits are of similar magnitude and are comparable to the value found for the dissociated form of phycoerythrin at high pH (ref. 5). Each red subunit contains the 565 mμ chromophore hidden when in the presence of PCMB and exposed upon the addition of glutathione. The better regeneration of the 565 mμ chromophore in the red subunit from *C. rubrum* appears to be due to its greater abundance. This red subunit also has a higher capability for assocation upon the addition of glutathione than does the red subunit from *P. cruentum*. This associated form of red subunit shows a greater degree of recovery of the 565 mμ chromophore.

It is known that phycoerythrin from *C. rubrum* contains 2 different kinds of pigment, namely, phycourobilin and phycoerythrobilin[6–8]. The purple subunit and perhaps the brown subunit may contain phycourobilin since both exhibit a major absorption maximum at about 500 mμ. The reason for the difference in their absorption characteristics between 500 and 600 mμ remains unknown. The red subunit probably contains both phycourobilin and phycoerythrobilin. The 500 mμ absorption has its origin in phycourobilin, while the 2 absorption bands at 540 and 565 mμ can be attributed to phycoerythrobilin[6–8]. In particular, since the 565 mμ band is sensitive to the effects of PCMB and to the degree of association of the subunit, one is led to conclude that there is a specific interaction involved between the phycoerythrobilin moiety and the apoprotein portion of the molecule giving rise to a structure absorbing at 565 mμ.

Recent studies[8–10] on N- and C-terminal residue analyses of phycoerythrin from *C. rubrum* indicate that this phycoerythrin may contain from 12 to 14 subunits in the molecule. VAUGHAN[4] suggests that this phycoerythrin may consist of 2 different kinds of subunit. This present investigation points out the possible existence of more than 2 different kinds of subunit in phycoerythrin from *C. rubrum*. The possibility of the presence of even smaller subunits with a different distribution of pigments is currently under investigation.

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