Association of Phycoerythrin and Phycocyanin: In Vitro Formation of a Functional Energy Transferring Phycobilisome Complex of *Porphyridium sordidum*†

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ABSTRACT: Functional in vitro association and dissociation of a phycobiliprotein complex, isolated from phycobilisomes of the red alga *Porphyridium sordidum*, were studied. The complex contained large bangiophyceaen phycoerythrin and cyanophytan phycocyanin in an equimolar ratio and had absorption maxima at 625, 567, and 550 nm and a shoulder at 495 nm. Emission at 655 nm (with excitation at 545 nm) from phycocyanin indicated functional coupling. The complex was stable over a wide buffer concentration range, and, notably, it was maximally stable in low phosphate, <0.01 M, unlike the phycobilisomes, which dissociate at this concentration. Its molecular weight was estimated to be ca. 510000, and by electron microscopy it was seen to consist of two units of similar size. The complex in 0.1 M phosphate was separated on a sucrose gradient into a homogeneous phycoerythrin band and a spectrally heterogeneous phycocyanin band. In vitro association of phycoerythrin and phycocyanin resulted in a complex with the same absorbance, emission, sedimentation, and molar pigment ratio as those of the native complex. The spectrally heterogeneous phycocyanin fractions from the dissociation gradient varied in the degree of association with phycoerythrin. Phycocyanin fractions absorbing from 622 to 633 nm exhibited high associability (>70%), whereas those with maxima at 617–620 nm had low associability (<30%). The presence of a 30000 molecular weight polypeptide accompanied high associability, where it was ca. 2-fold more prominent. It is suggested that this polypeptide is involved in complex formation and could serve either in the stabilization of the conformational state of cyanophytan phycocyanin or as a direct linker between phycobiliproteins.

The phycobiliproteins are light-harvesting pigments in red and blue-green algae (cyanobacteria) which contribute the energy absorbed primarily to photosystem II (Ley & Butler, 1977; Wang et al., 1977). Within the cell, they exist in phycobilisomes (PBS)[1] with a pigment arrangement consistent with maximum energy transfer [Bryant et al., 1979; Mörschel et al., 1977; reviewed in Gantt (1980)]. According to their absorption, most phycobiliproteins belong to one of three major classes: phycoerythrins (PE), with maxima ranging from 500 to 570 nm; phycocyanins (PC), with maxima at 610–635 nm; and allophycocyanins (APC), with maxima ranging from 617 to 670 nm. Their chromophores are covalently linked to apoproteins. Most of the phycobiliproteins consist of α and β subunits in a 1:1 ratio, while a few have γ subunits as well. Dependent on the phycobiliprotein, each subunit has from one (APC) to four (B-PE) chromophores (Bogorad, 1975; Glazer, 1977).

In all PBS, energetic uncoupling and subsequent dissociation between phycobiliproteins can be affected by changes in ionic conditions and temperature. Depending on the algal species, dissociation, as indicated by changes in fluorescence emission, was observed between APC and PC, PE and PC, and PC's and APC's (Gantt et al., 1979; Rigbi et al., 1980). Functionally coupled PE and PC complexes have been isolated from *Rhodella violacea* (Mörschel et al., 1977; Koller & Wehrmeyer, 1979), *Porphyridium sordidum* (Gantt et al., 1978), and *Nostoc* sp. (Canaani et al., 1980). This exhibited a fluorescence emission from PC (F650–655 nm) when excited through PE (545 nm). Such complexes could be further dissociated into their PE and PC components, exhibiting uncoupling of energy transfer by the individual phycobiliprotein emissions (F575–580 nm and F640–650 nm, respectively).

In this report, we describe the isolation from *P. sordidum* of a PE–PC complex that comprises a major portion of the PBS. From its separated components, we demonstrated an in vitro association of a biologically functional complex with efficient energy transfer from PE to PC. Except for a preliminary report (Gantt et al., 1978), to our knowledge this is the first time a complex has been formed which is structurally and functionally comparable to that obtained from PBS. This complex, together with a system from *Nostoc* sp. where complete reassociation of functionally intact PBS from larger dissociation products were obtained (Canaani et al., 1980), represents two model systems which should allow detailed analysis of PBS structure and phycobiliprotein interactions.

Materials and Methods

The culture of *Porphyridium sordidum* was a gift from Dr. F. Ott. Cells were grown in dilute sea water medium (Provasoli et al., 1957). The culture of the alga and the isolation of PBS in 0.75 M potassium phosphate (pH 6.8–7.0) were carried out as previously described (Gantt et al., 1979). Chemicals used were of reagent grade quality.

Preparation of the PE–PC Complex. Phycobiliprotein samples were always kept in potassium phosphate buffer, pH 6.8–7.0, in various molar concentrations. For dissociation, PBS were suspended to about 4 mg/mL in 5 mM buffer and dialyzed at ca. 23 °C overnight against the same buffer. Dilution of the sample was kept to a minimum. A 1-mL aliquot of sample was layered on a linear sucrose gradient (30 mL) composed of 0.1–1.0 M sucrose in 0.01 M buffer and 0.02%

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[1] Abbreviations used: APC, allophycocyanin; B-PE and b-PE, large and small bangiophyceaen phycoerythrin, respectively; C-PC, cyanophytan phycocyanin; PBS, phycobilisome(s); PE–PC complex, an aggregate consisting of C-PC and B-PE; $NaDodSO_4$, sodium dodecyl sulfate.

sodium azide. Centrifugation in a fixed-angle rotor at 175000*g* was carried out for 4 h at 20 °C. An alternate method for preparation of the PE–PC complex from dissociated PBS was by elution from a brushite column. A 4–5-mL PBS sample, dissociated as above, was layered on a brushite column (2.0 × 16 cm) equilibrated with 0.005 M phosphate. The PE–PC complex was eluted frontally in 0.005 M phosphate buffer. Its properties were the same as when collected by sucrose-gradient centrifugation, with the advantage that removal of sucrose was not necessary before proceeding, and small amounts of contaminating APC and possibly other proteins were removed.

The separation of the phycobiliproteins from the complex was carried out at 4 °C. The complex was suspended in 0.1 M buffer, and ca. 1.5 mg of protein was layered onto a linear gradient (30 mL) composed of 0.1–1.0 M sucrose in buffer and 0.02% sodium azide. The gradients were centrifuged in a fixed-angle rotor for 20 h at 94600*g*.

Phycobiliprotein samples were stored as precipitates obtained by addition of crystalline ammonium sulfate (75% w/v). For minimization of possible enzymatic degradation, proteolytic inhibitors (1.0 mM phenylsulfonyl fluoride, 1.0 mM benzamidine, and 5 mM ϵ-aminocaproic acid; Broglie et al., 1980) were present when sufficiently soluble in the buffer (>10 mM phosphate).

For molecular weight estimation of the PE–PC complex, a Sepharose 6B (Pharmacia) column (2 × 67 cm) was used. The column was equilibrated with 0.03 M buffer containing 0.02% sodium azide. The same buffer was used for the elution of the sample and marker proteins. Marker proteins were the following: B-PE (240000–260000) from *P. cruentum*; ferritin (480000–510000); β-galactosidase (510000–530000); thyroglobin (680000); L-arginine decarboxylase (850000).

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis on a slab gel apparatus (Bio-Rad Model 220) was performed essentially according to the system of Laemmli (1970) with some modification. The thickness of the slab gel was usually 1.5 mm, with a sample concentration of 50–80 μg/mL. A 2-cm stacking gel contained 6% (w/v) acrylamide, 0.15% *N*,*N*′-methylenebis(acrylamide), 0.15 M Tris-HCl (pH 6.8), and 0.1% NaDodSO$_4$. The running gel contained 20% (w/v) acrylamide, 0.15 M Tris-HCl (pH 7.8), and 0.1% NaDodSO$_4$. Both gels were polymerized with *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and ammonium persulfate. Tris–glycine (0.38 M) (pH 8.4) with 0.1% NaDodSO$_4$ was the electrode buffer.

The samples contained a final concentration of 2% NaDodSO$_4$, 1% β-mercaptoethanol, 0.05 M Tris-HCl, 0.002% bromophenol blue, and 10% glycerol. They were heated to 100 °C for 2 min before layering on the gels. Electrophoresis on the gels occurred for 15 h at 9 mA. The gels were then fixed in hot trichloroacetic acid for 20 min and stained with Coomassie blue R-250. Stained gel slices were scanned on a Gilford spectrophotometer, and the area under each curve was determined with digitized scans and Simpson's rule for integration.

Phycobiliproteins were estimated from known extinction coefficients (Gantt & Lipschultz, 1974; Glazer & Hixson, 1977; Bennett & Bogorad, 1973). Protein content was determined according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

Absorption spectra at room temperature were measured on a Cary 17 spectrophotometer. Fluorescence spectra were measured on an Aminco-Bowman fluorometer equipped with an automatic corrected spectrum attachment. For fluorescence spectra, the samples were diluted to a protein concentration of ca. 35 μg/mL or less, corresponding to an absorption of up to 0.1 absorbance unit at the λ_{max} of the predominant phycobiliprotein.

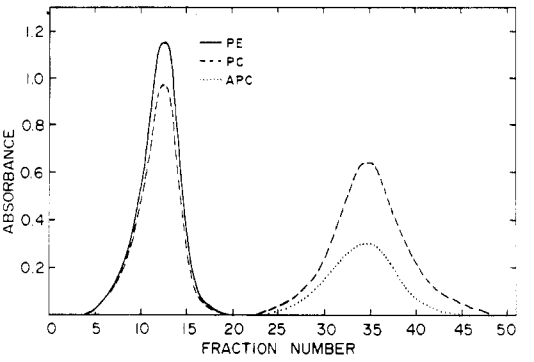

FIGURE 1: Fractionation profile of dissociated PBS on a sucrose gradient. The PE–PC complex was near the bottom (fractions 5–18), free PC and APC remained near the top (fractions 24–46) (corrected for spectral overlap). Phycobilisome dissociation was in 5 mM phosphate buffer overnight (23 °C). The sucrose gradient (0.1–1.0 M sucrose in 10 mM potassium phosphate buffer, pH 6.8, in 0.02% sodium azide) was centrifuged in a fixed-angle rotor at 175000*g* for 4 h at 20 °C.

Electron Microscopy. Samples were placed on grids, freshly exposed to glow discharge to enhance spreading, fixed for 1–3 min in 0.5% glutaraldehyde, rinsed with distilled water, and stained with 1% aqueous uranyl acetate for 1 min.

Results

Isolation and Characteristics of the PE–PC Complex. The PE–PC complex was isolated on a linear sucrose gradient from partially dissociated PBS. The complex recovered near the bottom of the sucrose gradient (Figure 1) contained ca. 99% of the PE of PBS and ca. 50% of the total PC. The free phycobiliproteins in the upper half of the sucrose gradient contained the remaining PC and all of the APC. The APC and PC were not energetically coupled because the emission peaks of PC and APC were separately resolvable, without any evidence of PC quenching. Occasionally, a small amount of PE (<1%) was found near the top of the gradient (fractions 44–45). Because of the small quantities, it is not known if it is comparable to b-PE, or if it is a degradation product of B-PE.

The native complex had major PE absorption maxima at 550 and 567 nm with a shoulder at ca. 495 nm (comparable to purified B-PE with λ_{max} at 545 and 563 nm) and a C-PC absorption maximum at 625 nm (Figure 2). It is significant that the emission peak of the complex was at 655 nm (PC) because it evidences the transfer of excitation energy (545 nm) from PE to PC, a measure of biological function. The complex was stable over a wide buffer concentration range (5 mM–0.75 M phosphate, pH 7.0). It was maximally stable in low ionic conditions and was routinely obtained and stored in 0.01 M potassium phosphate buffer. This is contrary to the isolation medium for PBS, or for several other phycobiliprotein complexes thus far studied. Most are stable at 0.5–0.75 M phosphate buffer (pH 6–8) and dissociate when the ionic strength is decreased (Gantt et al., 1979; Rigbi et al., 1980).

The prevalent images of the PE–PC complex in electron micrographs consisted of particles containing two units of similar size and shape (Figure 3). In side views, each of the two major units was further resolved into two smaller units (Figure 3B). The subunit structure of one of the major units is presumed to be B-PE $(\alpha\beta)_6\,\gamma$ and the other C-PC $(\alpha\beta)_6$. This assumption is also supported by total protein determi-

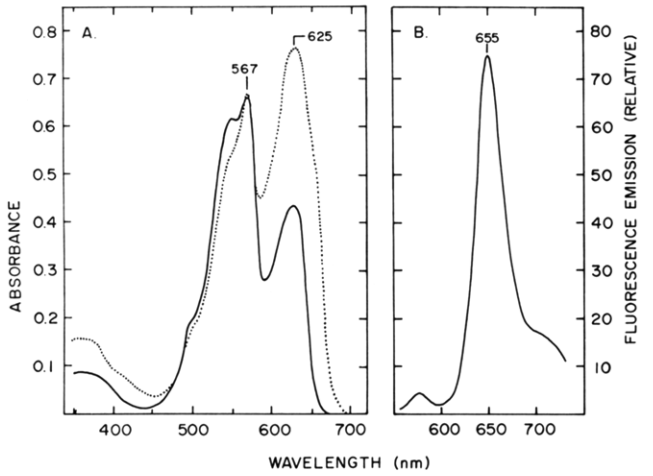

FIGURE 2: (A) Absorption spectra of PE–PC complex in 10 mM phosphate buffer, pH 6.8 (—), and PBS in 750 mM phosphate buffer, pH 6.8 (···), normalized at 565 nm. The maxima at 567 nm are primarily from PE, and at 625 nm from PC. (B) Fluorescence emission of the complex at 655 nm is from PC; the lower emission at 575 nm is from PE.

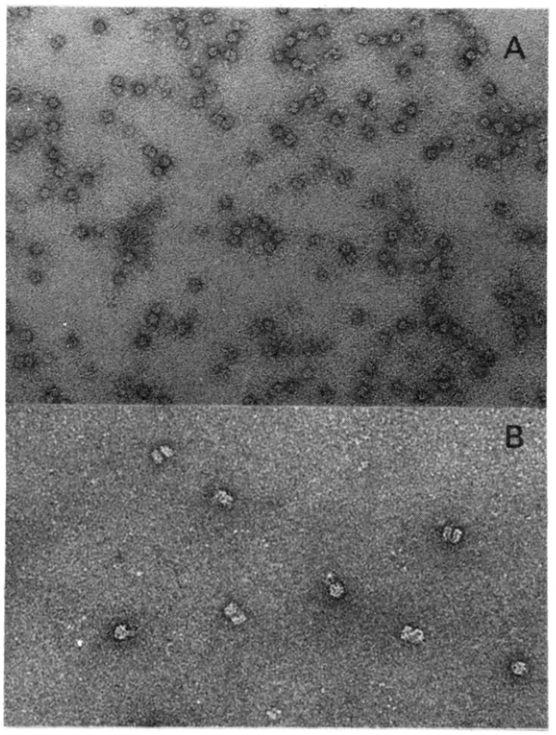

FIGURE 3: Electron micrographs of PE–PC complex preparation stained with uranyl acetate. (A) Many particles are seen to consist of two units with similar dimension (110 400×). (B) Enlargement of widely dispersed preparation (178 400×).

nations, showing that the PE and PC were present in about equivalent amounts. The apparent molecular weight of the complex by column chromatography was ca. 510 000, which allows for two hexamers, one B-PE (240 000–260 000) and one C-PC (ca. 250 000). These values closely agree with the molecular weights determined by gel filtration from other red algal sources (Gantt & Lipschultz, 1974; Koller & Wehrmeyer, 1979; Glazer & Hixson, 1977). By its pigment composition, this complex appears to correspond to a similar complex (505 000 molecular weight) isolated by Koller et al. (1978) from *Rhodella* PBS, which was obtained after the "tripartite unit" had lost one of its B-PE hexamers.

When the data on the size and composition of the complex are considered in terms of the total phycobiliprotein composition of the PBS, a probable structural relationship between them can be inferred. From the absorption spectrum of *P. sordidum* PBS (Figure 2A), the phycobiliprotein content has been determined to be ca. 14% APC, ca. 57% PC, and ca. 29% PE. Here, we have demonstrated that the PE–PC complex contains all the PE, one-half the PC, and no detectable APC. In addition, we have found that the PBS are hemidiscoidal in shape (electron micrograph, not shown) with a diameter of 44 ± 3 nm, a height of 32 ± 3 nm, and a thickness of ca. 10 nm (equivalent to the thickness of the complex). From this information, we can conclude that the complex is a major part of the PBS (ca. 58%) and that it is probably on the periphery, with PE comprising the outermost layer. Such an arrangement is consistent with the PBS structure in other PE-containing organisms (Gantt et al., 1976; Mörschel et al., 1977; Bryant et al., 1979).

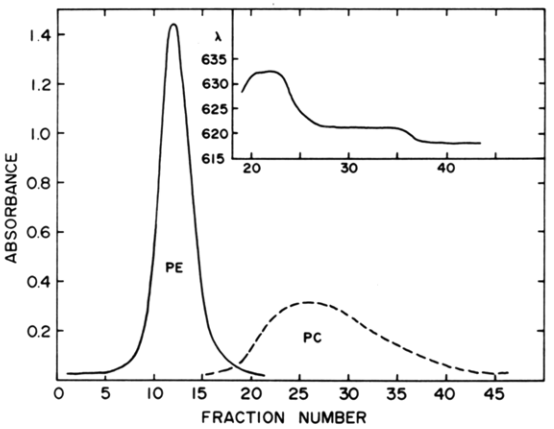

FIGURE 4: Fractionation of dissociated PE–PC complex on linear sucrose gradient (0.1–1.0 M sucrose in 100 mM phosphate buffer, pH 6.8, at 94600*g*, 20 h, 4 °C) (PE at OD 545; PC at OD 620). Bottom of gradient at left. The inset shows the change in the absorption maxima of the PC fractions.

Dissociation of the PE–PC Complex. Unlike most phycobiliprotein aggregates, this complex did not completely dissociate in low ionic strength buffer, nor even in distilled water at room temperature. Separation of the complex into its component biliproteins required centrifugation (94600*g*, 20 h) in 0.1 M phosphate on a linear sucrose gradient (0.1–1.0 M sucrose) at 4 °C (Figure 4). Phycoerythrin migrated toward the bottom of the gradient as a single symmetrical band. It was presumed to have the same molecular weight (240 000–260 000) as that of *P. cruentum* (Gantt & Lipschultz, 1974; Glazer & Hixson, 1977), because the sedimentation velocity of B-PE from the two species was the same. Phycocyanin, on the other hand, migrated in a wide zone and exhibited heterogeneity in its size and spectral characteristics (inset in Figure 4). The fastest migrating C-PC (fractions 20–25) absorbed maximally at ca. 633 nm (fluorescence at ca. 655 nm). The absorption maximum of the middle region was at ca. 622 nm (fractions 26–36). The C-PC near the top (fractions 37–44) had absorption peaks at ca. 617 nm (fluorescence 651 nm).

In Vitro Association of the Complex. Energetic coupling resulted when the PE and PC fractions were combined with one another. The functional integrity of this complex is identical with the native complex as shown by the fluorescence assay. Fractions of PC were mixed with PE in a ratio equivalent to that of the original complex, and association was determined by following the change in the fluorescence emission of PE at 575 nm (with excitation at 545 nm). The emission of PE decreased whereas that of PC increased (Figure 5), demonstrating transfer of energy. This energy transfer

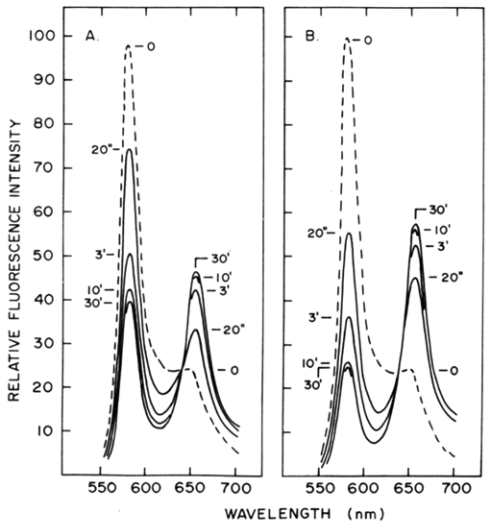

FIGURE 5: Time course of PE and PC association as measured by fluorescence (0.1 M phosphate buffer and ca. 0.5 M sucrose, pH 6.8, 20 °C). Excitation was at 545 nm. As association occurs, PE is quenched from the free state where the relative emission at 575 nm was 100% (t = 0). (A) A mixture of total combined PE and PC fractions as obtained from gradient shown in Figure 4. (B) Mixture of faster sedimenting PC (fractions 20–31) and PE.

Table I: Percentage of Association Occurring between Phycoerythrin and Phycocyanin in 30 min in 0.1 M Phosphate Buffer (pH 6.8) and 0.5 M Sucrose at 23 °C

experiment	top PC[a] plus PE (%)	bottom PC[b] plus PE (%)
1	30	68
2	21	74
3	29	75

[a] PC obtained from fractions 31–43, as in Figure 4. [b] PC obtained from fractions 20–31, as in Figure 4.

from PE to PC also correlated with association of the free biliproteins into a recoverable complex with a sedimentation identical with the native complex (see below). When the entire PE and PC contents from the gradient fractionations were mixed (Figure 5A), ca. 60% association occurred. However, when PC fractions 20–31 were mixed with PE, the association percentage was higher (Figure 5B). In 30 min, most of the association was completed because additional incubation for 12 h only yielded an 1–2% increase. Generally, the extent of association was enhanced by higher phycobiliprotein concentrations, relatively low ionic conditions (<0.1 M phosphate), and room temperature rather than 4 °C. However, a primary factor was the nature of the PC fraction. The most association (Table I) was obtained with the more rapidly sedimenting PC (fractions 20–31), while with more slowly sedimenting PC (fractions 31–43) less association occurred. The presence of a large excess of BSA (1 mg/mL) in the mixture did not alter the time or extent of association. Interestingly, B-PE from *P. cruentum* did not associate with PC under these conditions.

Reassociated complex had the same sedimentation velocity on linear sucrose gradients as did the native complex (conditions under Materials and Methods). Furthermore, both had the same molar ratios of PE and PC. Their absorption and emission spectra were virtually indistinguishable, as was their relatively low polarized fluorescence degree (J. Grabowski, unpublished experiments). Excess PE or excess PC did not alter the ratio of the pigments in the recovered associated complex. The polypeptide pattern on $NaDodSO_4$–polyacrylamide gel electrophoresis also did not differ significantly between the native and reassociated complex (see below).

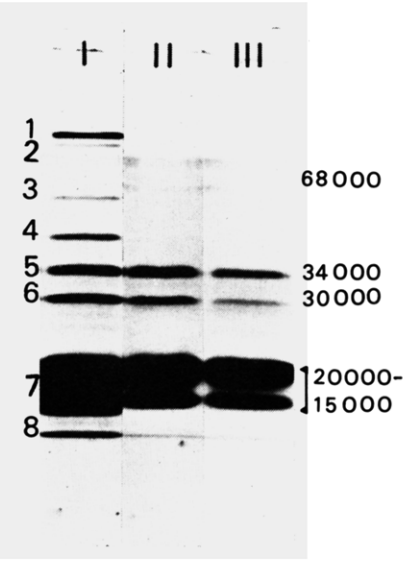

FIGURE 6: Polypeptide pattern on $NaDodSO_4$–polyacrylamide gel electrophoresis of PBS (I), native PE–PC complex (II), and in vitro associated PE–PC complex (III). Details under Materials and Methods. Relative molecular weight regions are indicated on right, and major polypeptide bands in PBS are on left.

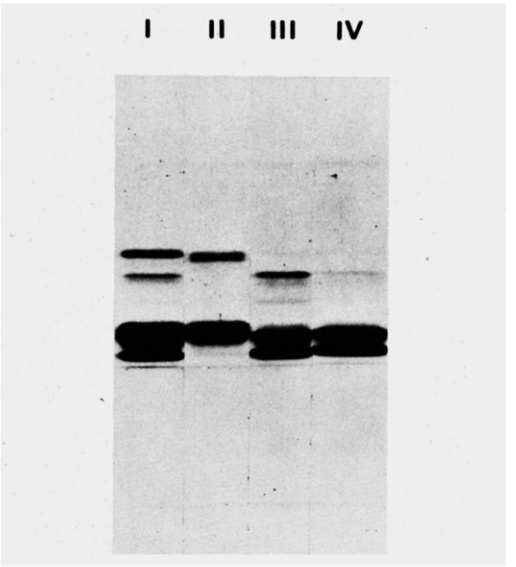

FIGURE 7: Polypeptide pattern on $NaDodSO_4$–polyacrylamide gel electrophoresis of reassociated PE–PC complex (I), PE (II), PC capable of high degree of association (III), and PC capable of low degree of association (IV).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of Phycobilisomes and Complex. Phycobilisomes of *Porphyridium sordidum* on $NaDodSO_4$–polyacrylamide gel electrophoresis, after Coomassie blue staining, exhibited several distinctive polypeptide regions and some minor bands (Figure 6). Prior to being stained, the second, third, fourth, sixth, and eighth bands were not colored. The uppermost (band 1), lane I in Figure 6, was blue. The fifth band was reddish and identified as the γ band characteristic of B-PE (Gantt & Lipschultz, 1974; Glazer & Hixson, 1977). The broad region near the bottom contained the colored α and β polypeptides of B-PE, C-PC, and APC. The thin lowermost band (ca. 11 000 molecular weight) was of an unknown nature.

The polypeptide pattern of the native PE–PC complex was characteristic of B-PE and C-PC. However, an additional polypeptide band with a molecular weight of ca. 30 000 was present. This was also a major component in PBS (compare Figure 6, lanes I and II). This polypeptide appeared to be necessary for complex formation, because it was present in the

native complex, as well as in the reassociated complex (Figure 6, lanes II and III). Furthermore, it seems to be preferentially associated with C-PC. Phycoerythrin isolated from dissociated complex, as in Figure 4, did not contain the 30 000 molecular weight polypeptide (Figure 7, lane II). This polypeptide was present in the C-PC fractions and was most prevalent in the highly recombinable (bottom PC) fractions (Figure 7, lane III). In C-PC fractions capable of the most association (75%), the 30 000 band comprised ca. 3–5% of the total Coomassie-stainable protein. In the fraction with relatively less association (30%), it comprised 1–2%.

Discussion

This report describes for the first time the in vitro association of two spectrally distinct phycobiliproteins. It is shown that the resulting complex efficiently transfers energy from PE to PC and is structurally and functionally comparable to the in vivo complex from *P. sordidum* PBS.

The results also provide evidence that a 30 000 molecular weight polypeptide in conjunction with PC is involved in association of PE and PC. This is in agreement with the postulation by Tandeau de Marsac & Cohen-Bazire (1977) that certain uncolored polypeptides present in PBS could function as linker proteins between phycobiliproteins. Since their report in 1977, uncolored polypeptides have been found in nearly all PBS examined (Bryant et al., 1979; Glazer et al., 1979; Redlinger & Gantt, 1980; Williams et al., 1980). In a recent report comparing wild-type and mutant cells, a 33 000 molecular weight polypeptide was suggested for stabilization of PC hexamers, while two other polypeptides (30 000 and 31 000 molecular weight) were believed to be involved with PE binding or with its stabilization (Williams et al., 1980).

At this time, the exact involvement of the 30 000 molecular weight polypeptide in the PE–PC complex is not known. It (a) could function as a direct link between PE and PC, or (b) may be involved in stabilizing a specific conformational state in PC, or could function in both roles. The 30 000 molecular weight polypeptide is obviously present only with PC and not with PE (Figure 7). Furthermore, the polypeptide is present in a greater amount in the faster sedimenting PC, which also exhibits the greatest associability with PE. We know that in the complex PC is about the size of a hexamer (ca. 250 000 molecular weight). The most direct interpretation of these observations is to assume that only large PC (presumably hexamer) associates into a functional complex with PE and that the polypeptide serves in the formation and/or stabilization of large PC. Further experiments are required to establish the role of the 30 000 molecular weight polypeptide. Should this polypeptide be found to serve in the stabilization of PC, the other similarly sized polypeptides, such as γ of B-PE and γ of APC I (Zilinskas et al., 1978), will have to be considered as serving a similar role.

Only one other red algal complex has been studied which also consists of B-PE and C-PC. It was obtained from the PBS of *Rhodella violacea*, and its components have been carefully characterized (Koller et al., 1978). This complex, however, does not contain any colorless polypeptides comparable to the 30 000 molecular weight polypeptide in *P. sordidum*. Thus, it seems that not all complexes have the same composition.

In PBS of *P. sordidum*, numerous polypeptides, in addition to the normal α and β polypeptides of the phycobiliproteins, are resolved in $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 6, lane I). It is expected that they are all important to the PBS structure and that their function will be elucidated in the future. However, the largest one (ca. 90 000 molecular weight) deserves special mention here, because it is considered as a probable anchor protein between the PBS and the thylakoid membrane, analogous to the findings in *P. cruentum*. In *P. cruentum*, a 95 000 molecular weight polypeptide has been shown to be present in the thylakoids, as well as in the PBS. The polypeptides from the two sources appear to be the same according to their tryptic digest patterns (Redlinger & Gantt, 1980). Such polypeptides are probably involved in the PBS–membrane attachment site.

In separation of PE and PC, it is of special interest to note that centrifugation itself also appeared to play a role in addition to low temperature (4 °C), by shifting the equilibrium as in other associating–dissociating systems (Harrington & Kegeles, 1973). In the current study, an apparent pressure effect has been put to advantage for improved separation and may be usefully explored in future characterization studies of phycobiliprotein complexes.

The fact that energy transfer can be correlated with association and dissociation of this heteromeric PE–PC complex provides a direct functional assay for studies on the phycobiliprotein linker proteins. This complex and similar ones from other algae will be extremely useful in studying the protein–protein interactions, chromophore orientation, and energy transfer from one chromophore type to another. In addition, capabilities exist for associating them into functional PBS when they are mixed with APC under specific conditions (Canaani et al., 1980). A much better understanding of the PBS structure should result from the more detailed characterizations of such phycobiliprotein complexes.

Added in Proof

Electrophoresis on long (ca. 26 cm) $NaDodSO_4$–polyacrylamide gradient gels, with greater resolution, yielded slightly higher molecular weight values. In Figure 6, band 5 had a molecular weight equivalent to 37 000 and band 6, to 31 000.

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Preparation of Photoreactive Derivatives of Glutathione and [9-(2-Mercaptotryptophan)]corticotropin by Selective Modification of the Sulfhydryl Group†

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ABSTRACT: The photoreactive arylsulfenyl chlorides 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl) have been used for the selective modification of thiol groups in glutathione and [Trp(SH)^9]corticotropin (ACTH). Both reagents reacted rapidly with both types of thiol groups to form unsymmetrical disulfides. The photoreactive derivatives of glutathione and [Trp(SH)^9]ACTH were stable to neutral and acidic conditions but were readily cleaved above pH 9 and by β-mercaptoethanol. Photolysis of the NAPS derivatives of [Trp(SH)^9]-ACTH at neutral pH resulted in the formation of covalently linked polymers and dimers which yielded monomer upon treatment with β-mercaptoethanol. Analysis of the amino acid composition of acid hydrolysates of photolyzed monomeric and dimeric products indicated a decrease in proline, valine, tyrosine, and phenylalanine.

Recently, we described the synthesis of two new photoreactive arylsulfenyl chlorides and their use for selective modification of tryptophan residues in peptides (Muramoto & Ramachandran, 1980). The reagents 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl)[1] and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl) were used to introduce a photoreactive group into the tryptophan residue of the pituitary hormone corticotropin (ACTH). The modified hormone 2,5-NAPS-Trp^9-ACTH was successfully employed for photoaffinity labeling of ACTH receptors on isolated rat adrenocortical cells (Ramachandran et al., 1980). Since arylsulfenyl chlorides are known to react with sulfhydryl groups also (Fontana et al., 1968), we have investigated the utility of 2,4-NAPS-Cl and 2,5-NAPS-Cl for the selective modification of sulfhydryl groups in peptides. In this article, we describe the selective modification of thiol groups of reduced glutathione and [Trp(SH)^9]ACTH as well as the photoreactivities of the modified peptides.

Materials and Methods

Highly purified porcine ACTH was prepared as previously described (Canova-Davis & Ramachandran, 1976). The syntheses of 2,4-NAPS-Cl and 2,5-NAPS-Cl have been reported previously (Muramoto & Ramachandran, 1980).

Preparation of the 2,4- and 2,5-NAPS Derivatives of Reduced Glutathione. To a solution of 120 mg (0.40 mmol) of reduced glutathione (Calbiochem) in 6 mL of glacial acetic acid was added 100 mg (0.44 mmol) of NAPS-Cl. The reaction mixture was stirred for 2 h at room temperature in the dark and poured into 100 mL of ethyl ether. The precipitate was filtered and washed with ethyl ether. NAPS-glutathione suspended in 50 mL of water was collected, washed with water and ethyl ether, and dried in vacuo: 2,4-NAPS-glutathione, yield, 120 mg (61%); mp 195 °C dec; TLC on silica gel G, R_f 0.36 in 1-butanol–acetic acid–water (4:1:1 v/v) (solvent A); IR (Nujol) 2090–2060 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{16}H_{19}N_7O_8S_2$ (M_r 501.5): C, 38.32; H, 3.82; N, 19.55. Found: C, 38.25; H, 4.01; N, 19.72. 2,5-NAPS-glutathione, yield, 180 mg (92%); mp 185 °C dec; TLC on silica gel G, R_f 0.36 in solvent A; IR (Nujol) 2100 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{16}H_{19}N_7O_8S_2$: C, 38.32; H, 3.82; N, 19.55. Found: C, 37.90; H, 3.99; N, 19.35.

Kinetics of Reaction of NAPS-chlorides with Reduced Glutathione. Equal volumes (2.5 mL) of a 20 mM solution of reduced glutathione in 90% acetic acid and 40 mM 2,4- or 2,5-NAPS-Cl in glacial acetic acid were mixed at 21 °C. At various times, 0.1 mL of the reaction mixture was added to 3 mL of water. Excess reagent was extracted with 3 mL of ethyl acetate. The aqueous phase (0.5 mL) was diluted with 1 mL of glacial acetic acid. The increase in absorbance at

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[1] Abbreviations used: ACTH, corticotropin; 2,4(5)-NAPS-Cl, 2-nitro-4(5)-azidophenylsulfenyl chloride; 2,4-DNPS-ACTH, [2,4-dinitrophenylsulfenyl-Trp^9]ACTH; [2,4(5)-NAPSS-Trp^9]ACTH, 2-nitro-4(5)-azidophenylsulfenyl derivatives of [Trp(SH)^9]ACTH; [Trp(SH)^9]corticotropin, [9-(2-mercapto)tryptophan)]corticotropin.

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