Role of the Colorless Polypeptides in Phycobilisome Reconstitution from Separated Phycobiliproteins

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ABSTRACT

A phycoerythrin (PE) and phycocyanin (PC) mixture was separated from allophycocyanin on calcium phosphate chromatography from completely dissociated phycobilisomes of the blue-green alga, Nostoc sp. After dialysis of the PE-PC mixture in 0.75 M potassium phosphate, pH 7, which allows reassociation of the dissociated pigment-proteins, complexes of PE and PC in a 2:1 m ratio (PE/PC complex) as well as complexes predominately of PC (PC/PE complex) were then separated by sedimentation on linear sucrose gradients. These complexes resemble the rods of intact phycobilisomes and transfer energy efficiently from PE to PC. They contain the Group II colorless polypeptides described by Tandeau de Marsac and Cohen-Bazire (1977 Proc Natl Acad Sci USA 74: 1635 61639). Phycobilisomes can be reconstituted by combining the allophycocyanin pool with (a) the PE-PC mixture, (b) the PE/PC complex, or (c) the PC/PE complex. Successful reconstitution is measured by absorption, fluorescence, circular dichroism, and electron microscopy. The major requirement for reconstitution is the 29-kilodalton colorless polypeptide. In its absence, no phycobilisomes are formed. It is the only colorless polypeptide common to both the PE/PC complex and the PC/PE complex, and appears to be the polypeptide responsible for rod attachment to the allophycocyanin. In addition, high phosphate concentrations and 20°C temperatures are needed for reconstitution.

PBsomes are aggregates of light-harvesting proteins which are attached to the thylakoid membranes of red and blue-green algae. PBsomes of Nostoc sp. are composed of three major types of phycobiliproteins: PE, PC, and APC. In addition to the major phycobiliproteins, PBsomes contain several colorless polypeptides which may function as structural components of the PBsome (14). Light energy absorbed by PE is efficiently transferred through PC to APC and then to the photosystem II reaction centers (4, 5).

The known path of excitation energy transfer through the PBsome has been incorporated into recent models describing the ultrastructure of the PBsome (1, 10, 19). A current model proposed by Bryant et al. (1) has several rods composed of PE and PC radiating out from a core of APC, with the PC in closest proximity to the APC and with the PE at the peripheral end of the rod. As indicated above, the association of PE and PC within the rods as well as the attachment of the rods to the core may require the presence of colorless polypeptides.

Canaani et al. (2) reported a reassembly of PBsomes from PBsomes partially dissociated in 0.4 M K-phosphate into two fractions separated on discontinuous sucrose gradients. We describe here experiments that show reconstitution of PBsomes from individually separated phycobiliproteins obtained by calcium phosphate chromatography of PBsomes completely dissociated in 10 mM K-phosphate. Necessary conditions are described for PBsome reconstitution, the most critical one being the presence of the 29 kd polypeptide of PBsomes.

MATERIALS AND METHODS

Nostoc sp. (strain Mac) was grown as previously described (13). Phycobilisomes were isolated as in Troxler et al. (15). PBsomes were suspended in 50 mM K-phosphate, pH 7, with 0.02% NaCl and 1 mM PMSF at a protein concentration of 15 mg/ml and dialyzed for 2 h with two changes against 10 mM K-phosphate, pH 7, with 1 mM PMSF. The dialysate was centrifuged for 30 min at 45,000g, and the supernatant was applied to a calcium phosphate column (2.5 × 45 cm) which had been equilibrated with 10 mM K-phosphate buffer, pH 7, containing 100 mM NaCl, 0.02% NaNO3, and 1 mM PMSF. The column was developed with the equilibration buffer which eluted PE and PC from the column, while the APC remained adsorbed to the calcium phosphate. This eluate from the brushite column containing a mixture of PE and PC and several colorless polypeptides was then dialyzed against 0.75 M K-phosphate, pH 7, with 0.02% sodium azide and 1 mM PMSF. After dialysis, this fraction is referred to as the 'PE-PC mixture'.

The PE-PC mixture was further fractionated by centrifugation on 0.15 to 0.80 M linear gradients of sucrose in 0.75 M K-phosphate, pH 7, at 40,000 rpm for 16 h in a Beckman SW40 rotor at 20°C. Separated were two phycobiliprotein-containing bands: a slower-sedimenting fraction that contained PC and PE in a ~4:1 m ratio ('PC/PE complex') and a faster-sedimenting fraction that contained PE to PC in a 2:1 m ratio ('PE/PC complex') (21).

The APC which remained adsorbed to the top of the brushite column was removed from the column and eluted from the brushite by suspension in 400 mM K-phosphate, pH 7, with 0.02% sodium azide and 1 mM PMSF. The APC was concentrated on an Amicon PM-10 ultrafilter cell and then dialyzed in 0.75 M K-phosphate. The resulting APC is referred to as the 'APC pool.'

Aliquots of the PE-PC mixture, PE/PC complex, and PC/PE complex were individually mixed with the pool of APC such that the ratio of protein in the PE-PC mixture to protein in the APC pool was 2:1 and the ratio of total protein in each of the individual complexes to the protein in the APC pool was 1:1. Protein con-

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45% of this PE-PC mixture and PE slab considered in these acrylamide stacking concentration with a biliprotein spectrofluorometer. On a made efficiency. pH 7, Hamamatsu TV carbon-coated grid.

Excess K-phosphate, K-phosphate, pH 7, were described dialysates in K-phosphate, K-phosphate, pH 7. These mixtures protein. These mixtures were dialyzed for 1 to 2 h against 0.75 M K-phosphate, pH 7. In addition, before application of the dissociated PBsomes to the brushite column, an aliquot was removed and dialyzed against 0.75 M K-phosphate, pH 7 (hereafter called 'directly reassociated PBsomes'). Each of the reconstituted PBsome dialysates described above were then centrifuged on the same discontinuous sucrose gradients ordinarily used for PBsome isolation as described earlier (15). Centrifugation was at 375,000g for 2 h at 20°C.

Absorption and fluorescence spectra were measured on reconstituted PBsomes which were removed from the 1.0 M sucrose layer. An aliquot was removed for negative staining; the remainder, after dilution with 0.75 M sodium phosphate, pH 7.0, was centrifuged at 375,000g for 4 h at 20°C; the resulting pellets were treated for gel electrophoresis.

For negative staining, PBsomes were prepared as in Williams et al. (17) with some modifications; PBsomes were diluted in 0.75 M K-phosphate, pH 7, with 0.05% bacitracin to a concentration of 20 μg/ml immediately before application to a Formvar-covered, carbon-coated grid. Excess sample was removed by suction with a fine-tipped Pasteur pipette. PBsomes were fixed on the grid for 5 to 10 min with 1% (v/v) glutaraldehyde in 0.75 M K-phosphate, pH 7, washed twice with 100 mM ammonium acetate, twice with 10 mM ammonium acetate, and negative stained with 1% (w/v) uranyl acetate or 1% (w/v) uranyl formate. Grids were examined in a Siemens Elmiskop 1A electron microscope operated at 80 kV.

Primary magnifications were ×40,000 to 70,000.

Absorption spectra were measured with a Cary 17D spectrophotometer. Fluorescence spectra were determined with an Amino-Bowman spectrophuorometer equipped with an R446S Hamamatsu TV photomultiplier tube. The band pass on the excitation side was 11 nm and 2.7 nm on the emission side. The spectra were uncorrected for lamp output and emission grating phototube efficiency. Circular dichroism measurements were made on a Cary 61 spectrophotometer in a 5-mm pathlength cell with a typical A of 1.1 at the maximum A. Phycobiliprotein concentration was determined spectrophotometrically using the simultaneous equations described by Bryant et al. (1). The sum of the biliprotein concentrations in each sample was used for total phycobiliprotein concentration in PBsomes or the PE-PC mixture for calculation of molecular ellipticity for circular dichroism measurements. The contribution of the colorless proteins was not considered in these calculations.

SDS-polyacrylamide gel electrophoresis on a 0.15 × 20 × 30-cm slab gel was done using the discontinuous buffer system of Laemmli (7) with a running gel of 12.5% acrylamide and a 5% acrylamide stacking gel. Samples were prepared for electrophoresis by dialysis against 10 mm sodium phosphate, pH 7, with 1 mM PMSF, followed by addition of an equal volume of sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 3% SDS, 5% β-mercaptoethanol, and 10% glycerol. Samples were heated for 5 min at 100°C. Electrophoresis was performed at a constant current of 25 mamp through the stacking gel and 40 mamp in the running gel. Gels were stained with 0.1% Coomassie brilliant blue R in 45% methanol and 10% acetic acid and were destained with 45% methanol and 10% acetic acid.

RESULTS AND DISCUSSION

The PE-PC mixture eluting from the brushite column consists of dissociated phycobiliproteins which are incapable of energy transfer from PE to PC. Dialysis of the mixture of PE and PC in 0.75 M K-phosphate, pH 7, results in the physical reassociation of the PE and PC into rod-like structures and restoration of energy transfer from PE to PC. Figure 1 shows the absorption spectrum of this PE-PC mixture in 0.75 M K-phosphate; the spectrum in 10 mM phosphate is quite similar with the only difference being in the decreased prominence of the PE shoulder at 550 nm. The fluorescence emission spectra of the dissociated PE-PC mixture in 10 mM phosphate buffer as eluted from the calcium phosphate column, and the PE-PC mixture after dialysis in 0.75 M K-phosphate, pH 7, are also shown in Figure 1. With excitation of PE by 530 nm light, reassociation of the PE with PC in high phosphate is seen by the decrease in fluorescence emission at 575 nm (due to PE) and a concomitant increase in fluorescence at 645 nm (due to energy transfer from PE to PC). Figure 1 also shows the absorption and fluorescence spectra of the pool of APC eluted from the brushite with 400 mM K-phosphate, pH 7. It has an absorption maximum at 650 nm and a fluorescence emission maximum at 665 nm. The ratio of absorbance at the visible wavelength maximum to 280 nm A of all biliprotein isolations is ~4 to 5, indicating the purity of the samples.

The absorption and fluorescence spectra of the PE/PC and PC/PE complexes separated from the PE-PC mixture on linear sucrose gradients are shown in Figure 2. The PE/PC complex contains about twice as much PE as PC on a molar basis, while the PC/PE complex is predominantly made up of PC (21). There is only a small amount of PE in the PC/PE complex which is seen as a shoulder at 570 nm in the spectrum of the PC/PE complex. The PE/PC complex contains two hexamers of PE and one hexamer of PC (21), and this is seen in the spectrum as a major absorption band with peaks at 550 and 570 nm (due to PE) and a lesser peak at 620 nm due to PC. Both of the complexes have fluorescence emission maxima at 645 nm when excited with 530 nm light, showing that they are transferring excitation energy from PE to PC.

For PBsome reconstitution experiments, various fractions of dissociated PBsomes were mixed with the APC pool and allowed to reassociate by dialyzing the mixtures in 0.75 M K-phosphate, pH 7, for 2 h at 20°C. After reassociation, the mixtures were layered on discontinuous sucrose gradients routinely employed in PBsome isolation and centrifuged at 375,000g for 2 h. On these
contain on which mixtures of gradients (tubes 3, 4, PBsomes mixture and the APC gradients, Neither the PE-PC mixture 1.0 the patterns fuged on "Materials and Methods"). (3) PBsomes reconstituted from PE-PC mixture and APC pool, (4) PBsomes reconstituted from PE/PC complex and APC pool, and (5) PBsomes reconstituted from PC/PE complex and APC pool. Conditions as described in Figure 3.

same 1.0 m step in the gradient as do freshly isolated PBsomes. The diffuse band in the 0.5 m sucrose layer represents unrecombined material which consists of less than 40% of the sample layered on the gradient.

The absorption spectra of the directly reassociated PBsomes and the PBsomes reconstituted from the separated PE-PC mixture and the APC pool closely correspond to the spectrum of PBsomes used for isolation of these components (Fig. 5A). The absorption spectra of the PBsomes reconstituted from PE/PC complex and APC and PE/PC/PE complex and APC (Fig. 5B) reflect the different pigment compositions of the complexes relative to freshly isolated PBsomes. Note the prominent shoulder of APC at 650 nm in the reconstituted material. Each of the reconstituted PBsomes with excitation of PE at 530 nm shows maximum fluorescence emission at 660 nm with a shoulder at 675 nm, which is indicative of energy transfer from PE to PC and finally to APC. The fluorescence emission of the PBsomes is at a slightly shorter wavelength as compared to the fluorescence emission maximum reported for Nostoc sp. PBsomes by Gantt et al. (3) which suggests that energy is not as efficiently transferred to the far-emitting APC I or APC B (15). However, this is also the case for PBsomes isolated in our laboratory under normal conditions (Fig. 5A), and is not simply a case of incomplete reassociation.

As seen in Figures 3 and 4, there are actually two separate PBsome bands on discontinuous sucrose gradients in the 1.0 m sucrose layer. The individual bands were separated and analyzed for their absorption and fluorescence properties and polypeptide compositions. The two PBsome bands within one gradient showed no obvious differences with respect to the above parameters. The lower of the two bands probably represents a fraction that contains aggregates of PBsomes, an effect of Triton treatment during PBsome isolation (5). These two bands have escaped detection earlier in our laboratory and in others which used Triton-X-100 in PBsome isolations in all likelihood by overloading of gradients with sample and the smaller volume of the 1.0 m sucrose step used in the gradients.

The circular dichroism spectra of freshly isolated PBsomes, directly reassociated PBsomes, PBsomes reconstituted from the PE/PC mixture and the APC pool, and the separate PE/PC mixture are shown in Figure 6. Note the similarity in the three PBsome spectra, particularly the structure at 670 and 650 nm conferred by APC which is absent from the PE/PC mixture. This is an independent measure of the successful reconstitution of the phycobilisomes. The circular dichroism spectra of Nostoc sp.
PBsomes show similarities with circular dichroism spectra of PBsomes from *Synechococcus* sp. 6301 (19) and *Fremyella diplosiphon* (11).

Electron micrographs of negatively stained, freshly isolated PBsomes of *Nostoc* sp. show that there are several rods radiating from a central core (Fig. 7A). Partial dissociation (perhaps due to incomplete glutaraldehyde fixation) of some of the freshly isolated PBsomes as well as some of the reconstituted PBsomes as a result of the negative staining procedure necessitated presentation of a representative micrograph of each sample in Figure 7. The number of intact PBsomes in the freshly isolated PBsomes was comparable to that of the reassociated PBsomes. The rods resemble the structures seen in negatively stained preparations of the PE-PC mixture and PE/PC complex (Fig. 7, E and F, respectively) and are composed of three discs about 6 nm thick and 11 nm in diameter. The directly reassociated PBsomes (Fig. 7B) as well as the PBsomes reconstituted from the PE-PC mixture and APC pool (Fig. 7C), PE/PC complex with the APC pool (Fig. 7D), and PC/PE complex and the APC pool (not shown) show the same morphological characteristics as those seen in the freshly isolated PBsomes. Four to five rods are visible on each PBsome with rod length varying from 18 to 20 nm. Elements of the APC core are visible in Figure 7C (indicated by arrow).

Recent models of PBsome ultrastructure have been proposed for a red alga (6, 10) and for several blue-green algae (1, 17) in which rods containing PE and PC radiate from a triangular core of APC. Koller et al. (6) have isolated 'tripartite units' from PBsomes which are most likely identical to the rods of the PBsomes. We believe that our PE-PC mixture represents PBsome rods and that the presence of a PE/PC complex and a PC/PE complex in the PE-PC mixture indicates that there may be two different populations of rods in PBsomes, one containing predominantly PE and one that is mostly PC. These two types of rods are synthesized under the culture conditions employed in this work. Growth of cells in cool-white light or in red light results in the
synthesis of only the PE/PC complex or only the PC/PE complex, respectively (B. A. Zilinskas, unpublished data).

Figure 8 is an SDS-polyacrylamide gel of reconstituted PBsomes and their components. In addition to the chromophore containing α- and β-subunits of phycobiliproteins (14 621 kd), the PBsomes (lane 2) also contain polypeptides of mol wts 95 and 80 kd. These are associated with APC as they are isolated only with the APC pool (lane 6). In addition, PBsomes and the PE-PC mixture (lane 3) contain four polypeptides of mol wts 29, 32, 34, and 34.5 kd. It can be seen that the 32- and 34-kd polypeptides are unique to the PE/PC complex (lane 4) and that the 34.5-kd polypeptide is unique to the PC/PE complex (lane 5). The polypeptide that both complexes and the PE-PC mixture have in common has a mol wt of 29 kd. This polypeptide appears to be more susceptible to proteolysis than the other polypeptides as it is preferentially broken down and is missing from polyacrylamide gel patterns (lane 13) within days after isolation. (The loss of the 29-kd polypeptide is more rapid in the absence of PMSF.)

Freshly isolated PBsomes and the various types of reconstituted PBsomes are seen in lanes 8 to 12 (Fig. 8). Note the presence of all the PBsome bands in the reconstituted PBsomes with the exception of the 95-kd polypeptide. The absence of the 95-kd polypeptide and the apparent increase in the amount of an 80-kd polypeptide in the reconstituted PBsomes is most likely a result of breakdown of the 95-kd polypeptide which occurred in the short time that elapsed (less than 24 h) between isolation of the PE-PC mixture, the APC pool, and the two complexes and reconstitution experiments. The partial breakdown of the 95-kd polypeptide did not prevent PBsome reconstitution, and, therefore, it is not involved in attachment of rods to the PBsome core. However, attempted reconstitution of PBsomes from a PE-PC mixture that lacks the 29-kd polypeptide (Fig. 8, lane 13) and the APC pool (Fig. 8, lane 6) was unsuccessful. Figure 9 shows a discontinuous sucrose gradient of uncombined PE-PC mixture that lacks the 29-kd polypeptide and the APC pool (tube 3).

The discovery of colorless polypeptides in PBsomes by Tandeau de Marsac and Cohen-Bazire (14) has generated much interest and speculation as to the possible function of these polypeptides. Tandeau de Marsac and Cohen-Bazire (14) first proposed that a possible role of the colorless polypeptides was to "position" the phycobiliproteins within the PBsomes. Evidence supporting this hypothesis has come from studies of changes in polypeptide patterns on polyacrylamide gels of PBsomes from chromatically adapted blue-green algae. Changes in the relative amounts of colorless polypeptides in response to variations in light quality have been observed (14)

Yamanaka and Glazer (18) observed that PBsome degradation in response to nitrogen starvation resulted in the breakdown of a 30-kd colorless PBsome polypeptide of *Synechococcus* 6301 as well as a decrease in phycocyanin content and sedimentation coefficient of the PBsomes. Williams et al. (17) working with mutants of *Synechocystis* 6701 with reduced amounts of PE and PC relative to APC observed that the PBsomes of the mutants lacked a polypeptide of mol wt 33.5 kd and contained reduced amounts of polypeptides of mol wt 31.5 and 30.5 kd as compared to wild type PBsomes. The mutant's PBsomes contained incomplete rods or had less than the usual number of the PE- and PC-containing rods. In other experiments with mutants of *Synechococcus* 6301, Yamanaka et al. (20) observed that the loss of two colorless polypeptides of mol wt 30 and 33 kd in the mutant corresponded to the loss of PC-containing rods of the PBsomes of this organism. Experiments of Lundell et al. (9) in which rods were made from purified hexameric PC and isolated colorless polypeptides suggest that the 30- and 33-kd polypeptides are involved in the assembly of PC into hexameric discs and rods of stacked discs, while the 27-kd polypeptide terminates rod assembly. Table I summarizes the mol wts of polypeptides believed to be associated with PBsome rods in three blue-green algae. The
absence of the 29-kd colorless polypeptides, PBsomes cannot be reconstituted from their components. Experiments in our laboratory have shown that dialysis of PBsomes in low concentrations of phosphate resulted in the breakdown of some of the colorless polypeptides of the phycobiliproteins. The presence of 1 mM PMSF or diisopropylfluorophosphate in the dialysis buffers prevented the breakdown of the polypeptides during dialysis in low phosphate (12). In particular, the stability of the 29-kd polypeptide in the PE-PC mixture was enhanced by the presence of PMSF.

Ruszkowski (12) also showed that limited proteolysis of PBsomes with trypsin resulted in specific breakdown of certain PBsome polypeptides, i.e. the 95-kd polypeptide associated with APC I, and the 29-kd polypeptide. These results suggest that breakdown of PBsome polypeptides is due to the presence of a protease. It appears that we may be coisolating a protease with the phycobilisomes, although there do not appear to be any extraneous polypeptides in our gels of phycobilisomes suggestive of its presence, unless it is masked in the region of the gel where the chromophore-containing subunits of the phycobiliproteins are found.

Canaani et al. (2) reported reassembly of PBsomes in Nostoc sp. from a PE-PC fraction and an APC fraction separated from partially dissociated PBsomes on a discontinuous sucrose gradient. They noted that a relatively long (16 h) dissociation of PBsomes in low phosphate prevented successful reassembly of PBsomes. It is likely that the long dialysis in low phosphate allowed a necessary component for PBsome reassembly to become broken down. Our data show this to be the 29-kd polypeptide.

We have established that the 29-kd polypeptide is a necessary condition for PBsome reconstitution. Its role in maintaining PBsome structure is most likely in attaching the PE-PC containing rods to the core of APC. This conclusion follows from several lines of evidence.

(a) It is the only polypeptide common to both the PE/PC complex and the PC/PE complex. These complexes morphologically resemble the rods of PBsomes (Fig. 7). The other polypeptides of the complexes are necessary for assembly and stabilization of the rods themselves.

(b) The 29-kd polypeptide is not required for association of PE and PC within the rods because PE-PC mixture and the individual complexes that lack the 29-kd polypeptide transfer energy efficiently from PE to PC (data not shown) and have similar sedimentation coefficients as the components containing the 29-kd polypeptide. Association of the PE and PC (or PC with PE) is not dependent on the 29-kd polypeptide.

(c) A close proximity of the 29-kd polypeptide and the APC core of PBsomes is indicated by the observation that some of our preparations of the APC pool show small amounts of the 29-kd polypeptide on polyacrylamide gels (lane 6 of gel).

Thus, the 29-kd polypeptide of the PE-PC rods is required for attachment of the rod to the APC core. In addition, recent experiments in our laboratory have shown that the entire APC pool is necessary for reconstitution of PBsomes. The APC pool can be separated into its four components (APC I, II, III, and B) (15); attempted PBsome reconstitution of the PE-PC rods with the
individual separated APC forms was not successful. This is not unexpected, as it may be possible to attach a rod to a single APC molecule with the addition of the PE-PC mixture to each of the separated APC forms; but, unless the other APC components of the core are present, the attachment of several rods to the APC core resulting in a whole PBsome cannot occur.

Last, we have previously shown (22) that hydrophobic interactions are necessary for structural and functional integrity of the PBsomes. It is interesting to note that Lundell et al. (9) have shown that the colorless polypeptides are hydrophobic proteins. Conditions which favor hydrophobic interactions may provide the optimal conditions for stabilization of PBsome structure by the colorless polypeptides. The requirement for 20°C temperature and high concentrations of phosphate buffer during protein isolation and PBsome reconstitution can be explained by strengthening of hydrophobic interactions by these conditions.

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