

Comparative Immunology of Algal Biliproteins

(Ouchterlony double diffusion/Cyanophyta/Rhodophyta/phycobiliproteins)

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Communicated by Emil L. Smith, October 1, 1971

ABSTRACT The three spectroscopically distinct classes of phycobiliproteins characteristic of the Cyanophyta and Rhodophyta—phycocyanins, allophycocyanins, and phycoerythrins—share no common antigenic determinants detectable by the Ouchterlony double diffusion technique. Each class of phycobiliprotein, from both Cyanophyta and Rhodophyta, possesses a strong determinant common to all members of that class. With respect to an antiserum directed against a specific cyanophytan biliprotein, all heterologous biliproteins of the same class are immunologically identical, as shown by the fact that absorption with a given heterologous antigen simultaneously eliminates crossreactions with other heterologous antigens. A cryptophytan phycoerythrin was found to be immunologically unrelated to any of the cyanophytan or rhodophytan biliproteins examined.

Previous studies on the immunology of algal phycobiliproteins (1-4) have shown substantial crossreactivity between the homologous pigments of both blue-green and red algae—a striking instance of an immunological relationship that spans the very wide gap between prokaryotic and eukaryotic cells. Nevertheless, there are contradictory reports with respect to several crucial points: notably, the immunological cross-reactivity of phyco- and allophycocyanins from a single organism (1, 3, 4), and of cyanophytan and rhodophytan phycoerythrins (2, 3). For this reason, a systematic reinvestigation of the problem, by the use of several immunological reference points for each type of phycobiliprotein, seemed desirable. We shall report here the results of a comparative study using antisera directed against the biliproteins of unicellular and filamentous blue-green algae, and against the phycoerythrin of a *Cryptomonas* sp.

MATERIALS AND METHODS

Sources of Phycobiliproteins. The purified phycobiliproteins used in this study were obtained from the biological sources shown in Table 1, which also shows the specific preparations used as immunizing antigens. It should be noted that the six strains of unicellular blue-green algae used as sources of phycobiliproteins were selected, from a much larger collection (5), on the basis of their diversity, particularly of the mean DNA base-composition.

Crude extracts prepared from four species of red algae were tested for immunological crossreactivity. The species were: *Porphyridium cruentum*, *P. aeruginum*, and *Asterocytis ramosa*

(class Bangiophyceae) and *Acrochaetium pectinatum* (class Florideophyceae).

Immunological Procedures. Antisera to the phycobiliproteins were obtained as described (7). Antisera specifically directed against *Aphanocapsa* sp. 6701 phycocyanin, *Synechococcus* sp. 6301 allophycocyanin, and *Fremyella* phycoerythrin, were absorbed with *Synechococcus* sp. 6301 phycocyanin, *Aphanocapsa* sp. 6701 allophycocyanin, and *Aphanocapsa* sp. 6701 phycoerythrin, respectively.

For immunodiffusion experiments, 1.7 g of Ionagar (Colab) was dissolved in 100 ml of distilled water, with the aid of gentle heating, and merthiolate was added to a final concentration of 0.02%. This solution was then mixed with 100 ml of barbital buffer at pH 8.2 (10.3 g of sodium 5,5-diethylbarbiturate, 1.84 g of diethylbarbituric acid, 6.81 g of sodium acetate trihydrate, and 950 ml of distilled water; the pH was adjusted to 8.2 with either NaOH or HCl).

In tabulating double immunodiffusion patterns (see Table 2), no distinction has been made between reactions of identity and reactions with spurring: both are recorded as “+”. Insofar as possible, pure antigens were used in double diffusion experiments. However, individual phycobiliproteins were purified from only one of the red algae examined, *Cyanidium*;

TABLE 1. Sources of purified algal biliproteins

Organism	Reference	Biliproteins purified*
Unicellular blue-green algae		
<i>Synechococcus</i> 6301	(5)	PC, AP
<i>Synechococcus</i> 6312	(5)	PC
<i>Synechococcus</i> 6307	(5)	PC
<i>Synechococcus</i> 6901	(5)	PC
<i>Aphanocapsa</i> 6701	(5)	PC, AP, PE
<i>Aphanocapsa</i> 6714	(5)	PC
Filamentous blue-green alga		
<i>Fremyella diplosiphon</i> †	(4)	PC, AP, PE
Eukaryotic algae		
<i>Cyanidium caldarium</i> ‡		PC, AP
<i>Cryptomonas</i> sp.	(6)	PE

* PC = phycocyanin; AP = allophycocyanin; PE = phycoerythrin. All pigments purified as described in ref. 7, except for those of *Fremyella diplosiphon* (ref. 4) and *Cryptomonas* sp. (ref. 6). Immunizing antigens are shown in boldface type.

† Purified phycobiliproteins and antisera kindly furnished by Drs. A. Bennett and L. Bogorad.

‡ Axenic strain, isolated at Berkeley by Mr. J. Waterbury.

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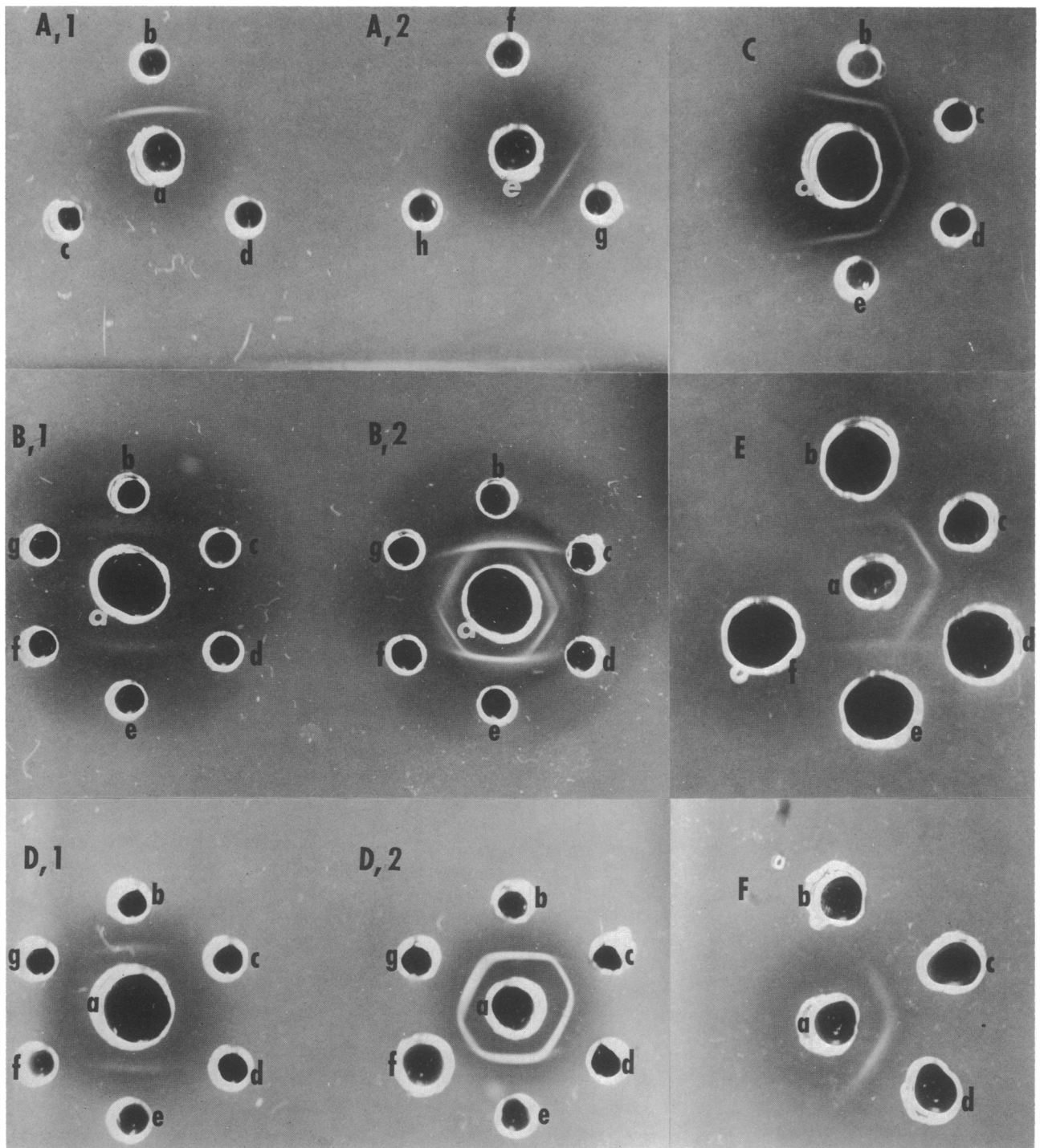


FIG. 1. Representative Ouchterlony double diffusion experiments with phycobiliproteins and antiphycobiliproteins. In all experiments shown, antibody wells contained 10–50 μ l of undiluted antiserum, and antigen wells contained 10–50 μ g of protein.

(A,1) Well *a* contains antiserum to *Aphanocapsa* strain 6701 AP; wells *b*, *c*, and *d* contain *Aphanocapsa* AP, PE, and PC, respectively. (A,2) Well *e* contains antiserum to *Aphanocapsa* PC; wells *f*, *g*, and *h* contain *Aphanocapsa* AP, PC, and PE, respectively.

(B) an experiment with antiserum to *Aphanocapsa* strain 6701 PC, unabsorbed and after absorption with a heterologous antigen, *Synechococcus* strain 6301 PC. Well *a* in *B,1* contains absorbed antiserum, and in *B,2*, well *a* contains unabsorbed antiserum. In both *B,1* and *B,2*, antigen wells *b* and *e* contain *Aphanocapsa* strain 6701 PC and antigen wells *c*, *d*, *f*, and *g* contain the PC of *Synechococcus* strains 6312, 6301, 6307, and 6901, respectively. The hazy diffusion patterns in *B,2* reflect overloading with antiserum, necessary to facilitate demonstration of the weak residual crossreaction with homologous antigen in *B,1*.

(C) Well *a* contains antiserum to *Aphanocapsa* strain 6701 PC. Wells *b* and *e* contain *Aphanocapsa* PC; wells *c* and *d*, contain *Synechococcus* strain 6301 PC and *Fremyella diplosiphon* PC, respectively.

(D) an experiment with antiserum to *Synechococcus* strain 6301 AP, unabsorbed and after absorption with a heterologous antigen, *Aphanocapsa* strain 6701 AP. Well *a* in *D,1* contains absorbed antiserum, and in *D,2*, well *a* contains unabsorbed antiserum. In both *D,1*

TABLE 2. The crossreactions of some cyanophytan, rhodophytan, and cryptophytan phycobiliproteins with nine specific antisera as determined by the Ouchterlony double diffusion technique

Antiserum to	Biliproteins* from																		
	Prokaryotes										Eukaryotes								
	PC	AP	PC	AP	PE	PC	AP	PE	PC	PC	PC	PC	PC	AP	PE				
	<i>Synechococcus</i> sp. 6301	<i>Synechococcus</i> sp. 6301	<i>Aphanocapsa</i> sp. 6701	<i>Aphanocapsa</i> sp. 6701	<i>Aphanocapsa</i> sp. 6701	<i>Fremyella diplosiphon</i>	<i>Fremyella diplosiphon</i>	<i>Fremyella diplosiphon</i>	<i>Synechococcus</i> sp. 6307	<i>Synechococcus</i> sp. 6312	<i>Synechococcus</i> sp. 6901	<i>Aphanocapsa</i> sp. 6714	<i>Cyanidium caldarium</i>	<i>Cyanidium caldarium</i>	<i>Porphyridium cruentum</i> †	<i>Porphyridium aerugineum</i> †	<i>Asterocytis ramosa</i> †	<i>Acrochaetium pectinatum</i> †	<i>Cryptomonas</i> sp.
<i>Synechococcus</i> sp. 6301	PC	+	-†	+	-	-	+	-	-	×	×	×	+	×	+	+	+	-	-
<i>Synechococcus</i> sp. 6301	AP	-	+	-	+	-	-	+	-	×	×	×	×	+	+	+	+	-	-
<i>Aphanocapsa</i> sp. 6701	PC	+	-	+	-	-	+	-	+	+	+	+	+	×	+	+	+	-	-
<i>Aphanocapsa</i> sp. 6701	AP	-	+	-	+	-	-	+	-	×	×	×	×	×	+	+	+	-	-
<i>Aphanocapsa</i> sp. 6701	PE	-	-	-	-	+	-	+	×	×	×	×	×	×	+	×	+	-	-
<i>Fremyella diplosiphon</i>	PC	+	-	+	-	-	+	-	-	×	×	×	×	×	+	×	+	-	-
<i>Fremyella diplosiphon</i>	AP	-	+	-	+	-	-	+	-	×	×	×	×	×	+	+	+	-	-
<i>Fremyella diplosiphon</i>	PE	-	-	-	-	+	-	+	×	×	×	×	×	×	+	×	+	-	-
<i>Cryptomonas</i> sp.	PE	×	×	×	×	-	×	×	×	×	×	×	×	×	-	×	×	-	+

* Abbreviations are as in footnote to Table 1; † crude extract, supernatant after high-speed centrifugation of broken cells; ‡ -, no precipitate was detected; § ×, not done.

crude extracts were tested from four other representatives of this group. Most of the particulate material in these extracts was removed by high-speed centrifugation before use in the immunodiffusion test. Although a visible crossreaction obtained with a crude extract is significant, failures to crossreact do not provide unequivocal proof that crossreacting material is absent from the extract, since the relative concentrations of the various phycobiliproteins in these samples were not determined.

RESULTS AND DISCUSSION

Results are summarized in Table 2; some salient points are illustrated by photographs (Fig. 1). From these data, the following conclusions can be derived.

(a) No immunological relationships are detectable by the double diffusion technique among the three classes of cyanophytan phycobiliproteins, even when the three antigens are derived from a single strain, and tested against the three corresponding homologous antisera (Fig. 1A). The noncross-reactivity of rhodophytan phycobiliproteins has been demonstrated by Bogorad (1) for the phycocyanin and allophycocyanin of *Cyanidium caldarium*, and by Vaughan (2) for the phycocyanin and phycoerythrin of *Ceramium rubrum*.

and D,2, antigen wells b and e contain *Synechococcus* sp. 6301 AP, wells c, d, f, and g contain the AP of *Cyanidium caldarium*, *Aphanocapsa* sp. 6701, *Porphyridium cruentum* (crude extract), and *Fremyella diplosiphon*. The amounts of antiserum and antigens were identical in D,1 and D,2.

(E) reactions of a crude extract of *Porphyridium cruentum* (well a) with a series of different antisera: b, to *Aphanocapsa* sp. 6701 AP; c, to *Synechococcus* sp. 6301 AP; d, to *Fremyella* AP; e, to *Fremyella* PE; f, to *Cryptomonas* sp. PE. Note reactions of identity with anti-APs in b, c, and d.

(F) reactions of an antiserum to *Synechococcus* strain 6301 PC (well a) with crude extracts of three red algae: *Acrochaetium pectinatum* (well b), *Asterocytis ramosa* (well c), and *Porphyridium aerugineum* (well d).

Abbreviations are the same as indicated in the footnote to Table 1.

(b) Each class of cyanophytan phycobiliprotein possesses a strong determinant (or determinants), shared by all spectroscopically recognizable members of that class, irrespective of the blue-green alga from which they are derived (Fig. 1B and C).

(c) This determinant (or determinants) is also shared by the spectroscopically homologous phycobiliproteins of red algae that belong to the two sub-classes Bangiophyceae and Floridophyceae (Figs. 1D, E, and F).

(d) The phycoerythrin of a cryptomonad is immunologically unrelated to any phycobiliproteins of the blue-green algae (6) or red algae (ref. 6; see also Fig. 1E). *Cryptomonas* phycocyanin has also been shown not to crossreact with antisera to cyanophytan and rhodophytan phycocyanins (3).

Two independent types of evidence support the postulated existence of a major antigenic determinant that is common to all cyanophytan and rhodophytan biliproteins of a given class. Firstly, if an antiserum to a specific cyanophytan phycobiliprotein is absorbed with a heterologous antigen from a blue-green alga that belongs to a different genus, all other heterologous crossreactions are simultaneously eliminated. The absorbed antiserum still gives a relatively weak crossreaction with the homologous antigen. This is specifically documented

(Fig. 1B and D) for phycocyanins and allophycocyanins, of which we possessed a relatively large number of pure preparations that were derived from different biological sources. Comparable results, but with less extensive comparative material, were obtained for phycoerythrins. The remarkable conservation of the antigenicity of phycobiliproteins is also documented by the data of Vaughan on rhodophytan phycoerythrins (2). Two antisera, directed against the phycoerythrins of one member of each rhodophytan subclass, both gave reactions of identity or near-identity with the phycoerythrins of 15 other red algae, which spanned the whole range of rhodophytan structural diversity.

Secondly, we have observed (Fig. 1E) that a crude extract of the red alga *Porphyridium cruentum* gives crossreactions of identity (no spurs) when tested simultaneously against three antisera directed against the allophycocyanins of three blue-green algae that are not taxonomically closely related to one another (*Synechococcus* 6301, *Aphanocapsa* 6701, and *Fremyella diplosiphon*).

The extreme immunological conservation of the phycobiliproteins could, of course, have a trivial explanation: namely, that antibodies are directed primarily against the bilin that serves as the chromophore. There are, however, strong arguments against this interpretation. In the first place, it has been shown (8–10) that the chromophores obtained by chemical cleavage from the phycocyanins and allophycocyanins of both blue-green and red algae are identical. Since the two classes of native proteins are totally noncrossreacting, the prosthetic group *per se* cannot be the major determinant. An elegant experiment of Vaughan (2) with the phycoerythrin of *Ceramium rubrum* also supports this conclusion: a tryptic digest of the protein, even at a 20-fold molar excess, did not inhibit either the homologous or heterologous antigen-antibody reactions. Vaughan (2) also showed that the chromophore of phycoerythrin is inaccessible to titration in the native protein, but becomes accessible when the protein is denatured. This suggests that the chromophore of the native protein has a limited exposure to the solvent, and by extension, to antibody molecules. The antigen-antibody reaction does not appear to affect the fluorescence of the phycobiliproteins. None of this evidence can be taken to mean that the chromophore has no role in determining the antigenic properties of a phycobiliprotein, but all the evidence suggests that the chromophore does not act as a simple haptenic determinant. The antigen-antibody reaction must therefore result from some higher order of structural complexity (a specific surface configuration

of the native biliprotein) that is class-specific, and may well be determined by structural parameters that also determine the specific spectroscopic properties of the molecule.

At the outset of this work, we hoped that the degree of immunological divergence within each spectroscopically distinct class of phycobiliproteins might suffice to permit an assessment of the broad evolutionary relationships among the very diverse algae, prokaryotic and eukaryotic, that contain such pigments. It is now evident that the degree of immunological divergence of the phycobiliproteins among the Cyanophyta and Rhodophyta is too limited to permit such analyses. On the other hand, the fact that antibodies directed against the major antigenic determinant(s) common to each spectroscopically distinct class of phycobiliproteins can be removed by absorption with a heterologous member of the same class makes it easy to prepare absorbed antisera that contain a small residuum of antibodies specifically directed against the homologous antigen. Such absorbed antisera will certainly be of considerable taxonomic value, since their use will permit the ready assignment of all blue-green and red algae to a series of clusters, each characterized by possession of immunologically identical phycobiliproteins.

We are deeply indebted to Mrs. Anajane Smith for the preparation of antisera and the performance of numerous immunodiffusion experiments. We also thank Drs. A. Bennett and L. Bogorad for the provision of purified phycobiliproteins and antisera, and for making available to us some of their own unpublished data, and Dr. John West for the provision of cultured material of *Acrochaetium pectinatum*. The work was supported by grant GB-17517 from the National Science Foundation to Prof. M. Doudoroff. A. N. G. was a fellow of the J. S. Guggenheim Memorial Foundation.

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