Immuonochemistry on Cryptomonad Biliproteins

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ABSTRACT

A survey is made of the immunochemical behavior of four of the six known types of cryptomonad biliproteins: phycoeyanins 612 and 645 and phycoerythrins 545 and 566. They were compared both among themselves and to selected biliproteins isolated from blue-green and red algae. All the cryptomonad biliproteins were shown to be closely related to each other by Ouchterlony double diffusion techniques. An antigenic relationship among all the cryptomonad biliproteins and B-phycoerythrin (red alga) and C-phycoerythrin (blue-green alga) was established. Only a very marginal cross-reactivity was found between C-phycoeryanin (blue-green algae) and the cryptomonad biliproteins. These results suggest a common ancestor for the photosynthetic units of all three biliprotein-containing phyla.

Biliproteins are light harvesting and excitation energy transfer chromoproteins that function as accessory pigments for PSII in the photosynthesis of blue-green (cyanobacterial), red, and cryptomonad algae. Cryptomonad biliproteins are unusual in that they apparently do not form phycobilisomes and in the cryptomonads no analog of allophycocyanin has yet been detected. There are six biliproteins in the cryptomonads, phycoeyanins 612, 630, 645, and phycoerythrins 545, 555, 566, with only one type usually found in each alga.

Immunochemical studies on biliproteins have greatly emphasized blue-green and red algal examples. By 1967 four publications had established in a very extensive and decisive manner three basic rules governing the immunochemical behavior of these two types of algal biliproteins (1–3, 29). These three generalizations are: all phycoeyanins from both blue-green (procar- yotes) or red (eucaryotes) algae are immunochemically very similar; all phycoerythrins of all spectral types (C-, R-, B-) are immunochemically closely related whether they are from blue-green or red algae; no phycoerythrin is related immunochemically to any phycoerythrin. This last rule holds even when the phycoerythrin and phycoerythrin are isolated from the same alga. These first two results were most important because of the great differences in structure between procaroyctic blue-green algae and eucaryotic red algae. The immunochemical results then established the principle that, although major cellular changes have occurred in the evolution from procaroyctes to eucaryotes, the individual proteins could remain virtually unaltered. Subsequent research has shown that the two subunits of phycoerythrin are immunochemically related (27) and the properties of allophycocyanin have been investigated (12).

Research on the immunochemistry of cryptomonad biliproteins, has been much less extensive and then usually only a fragment of a larger study. It is therefore not too surprising that unlike the comparison of blue-green and red algal biliproteins, cryptomonad results have been so far perhaps inconclusive and controversial (2, 4, 12, 13, 16, 26, 29). We, therefore, undertook an immunochemical investigation using Ouchterlony double-diffusion and four different cryptomonad biliproteins. Two different phycoerythrins and two different phycoeyanins were compared among themselves using several different antiseras. For one, phycoeyanin 612, this is the first immunochemical study. They were then tested against selected biliproteins from blue-green (C- phyocyanin and C-phycoerythrin) and red algae (B-phycoery- thrin).

EXPERIMENTAL

Biliproteins were isolated and purified from cryptomonads, phycoeyanin 612–Hemiselmis virescens, phycoeyanin 645–Chroomonas species, and phycoerythrin 545–Rhodomonas lens, and phycoerythrin 566–Cryptomonas ovata, as described previously (14). The cryptomonad proteins were extensively purified by (NH4)2SO4 fractionation and gel filtration on Sepharose 4B and Ultrogel AcA54. The protein purity was established by the ratio of the absorbances of visible absorption maximum to 280 nm and by SDS gel electrophoresis. C-Phycocyanin was isolated by lyosome treatment of Phormidium luridum and purified by fractionation using (NH4)2SO4. C-Phycocyanin (P. persicium and Calothrix membranes) and B-phycoerythrin, and allophycocyanin (Porphyridium cruentum) were purified by (NH4)2SO4 fractionation followed by chromatography on hydroxyapatite (Bio-Rad, Richmond, CA) using a phosphate gradient. The purity of the biliproteins from cyanobacteria and red algae was monitored by the ratio of absorption at the visible maximum to absorption at 280 nm.

Antiserum was derived by injecting purified biliproteins together with Freund's complete adjuvant (Difco, Detroit, MI) in the neck area of rabbits. Prior to the first injection some blood was taken from a vein in the ear to serve as a control. All such prebleeds ultimately proved to be negative in tests versus biliproteins. Our immunization protocol called for two injections of 2 to 4 mg of protein each at a 1 month interval. A week following the second exposure the animals were anesthetized with ketamine hydrochloride (Bristol, Syracuse, NY), and a maximal amount of blood withdrawn via cardiac puncture. Sera were prepared by centrifugation and stored frozen. In some cases the serum was fractionated by (NH4)2SO4 precipitation prior to use. Antiserum was prepared against the following: phycoeyanin 612 (H. virescens); phycoerythrin 545 (R. lens), phycoerythrin 645 (Chroomonas sp.); phycoerythrin 566 (C. ovata); B-phycoerythrin (P. cruentum); C-phycoerythrin (Synecococcus lipidus); C-phycoerythrin (P. persicium). Several rabbits were used for each type of antigen.

Ouchterlony double diffusion experiments were performed in

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AB = anti phycoerythrin 566

Fig. 1. Ouchterlony double diffusion using antisera (AB) against cryptomonad phycoerythrin 566 and cryptomonad antigens. In this Figure and in those subsequent the following symbols have been used for the antigens: BPE, B-phycoerythrin (P. cruentum); CPC, C-phycocyanin (P. luridum); CPE, C-phycoerythrin (P. persicinum); CPC, C-phycoerythrin (C. membranacea); 545, phycoerythrin 545 (R. lens); 566, phycoerythrin 566 (C. ovata); 645, phycocyanin 645 (Chroomonas sp.); 612, phycocyanin 612 (H. virescens).

AB = anti phycoerythrin 545

Fig. 2. Ouchterlony double diffusion using antiserum against cryptomonad phycoerythrin 545 and cryptomonad antigens.

AB = anti phycocyanin 645

Fig. 3. Ouchterlony double diffusion using antiserum against cryptomonad phycocyanin 645 and cryptomonad antigens.

### Table 1. Amino Acid Compositions of Cryptomonads Phycoerythrin 566 and Phycocyanin 612

<table>
<thead>
<tr>
<th></th>
<th>Phycoerythrin 566</th>
<th>Phycocyanin 645</th>
<th>Phycocyanin 612</th>
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<tbody>
<tr>
<td>Amino Acid</td>
<td>residues per 24,000</td>
<td>residues per 24,000</td>
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<tr>
<td>Asp</td>
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<td>17.2</td>
</tr>
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</tr>
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<tr>
<td>Cys</td>
<td>6.3</td>
<td>11.1</td>
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</table>

* Values from literature: MacColl et al. (16).  
* * Extrapolated to zero hydrolysis time.  
* * Value of longest hydrolysis time.

1% agar prepared in pH 6.0 sodium phosphate buffer. All antigen were prepared in the same buffer. Usually 100 µl of antiserum or antigen were placed in each well and the antiserum was always in the center surrounded by regularly spaced antigen wells. The protein content in each antigen well varied from 0.02 to 0.20 mg. Some of the Ouchterlony results resemble those published earlier but are included for completeness and because of the lack of agreement on the significance of the earliest data.

Amino acid analyses were performed after hydrolysis of the proteins in a vacuum at 110°C in 6 N HCl for 24, 48, and 72 h. Cysteine was determined separately as cysteic acid by treatment during acid hydrolysis with DMSO. After evaporation to dryness, hydrolyzed samples were dissolved in 0.2 M sodium citrate buffer (pH 3.2). Amino acids were separated and quantitated using a Beckman 119 CL amino acid analyzer and the Beckman single column system. Small unidentified peaks were frequently ob-
RESULTS AND DISCUSSION

Four different cryptomonad biliproteins and several antisera are used to establish their antigenic relatedness. Antisera to phycoerythrin 566 (Fig. 1) and phycoerythrin 545 (Fig. 2) clearly show extremely close antigenicity among all the cryptomonad biliproteins. This concept is further supported by Ouchterlony results using antiserum against phycocyanin 645 (Fig. 3). This result is surprising when compared to the heavily studied phycoerythins and phycocyanins from blue-green and red algae which are not antigenically related (2).

The amino acid composition of phycocyanin 612 was obtained from (Table 1). Its composition is compared to previously published data on phycocyanin 645 and phycoerythrin 545. It is noted that these three proteins have general similarities in their amino acid compositions.

Also controversial have been experiments designed to test if
crytomonad biliproteins when also are (spurring) is cryptomonad imments with result but this from the blue-green unrelated scheme in crograph biliproteins cryptomonad are roplast The from blue-green algae. C-phycoerythrin (blue-green algae) marked. Electron micrographs from other groups have clearly demonstrated this aspect of the cryptomonad chloroplast, and this micrograph is included as another example.

cryptomonad biliproteins are immunochemically related to those from blue-green and red algae. Earlier results showed no cross-reactivity (12, 13, 29). This result led to formulation of an evolutionary scheme in which the cryptomonads were completely unrelated or at best diverged at an extremely early time from the blue-green algae or a common ancestral organism (10). Ouchterlony experiments using antisera against cryptomonad phycoerythrins, however, offer very strong contradictory results (Figs. 4 and 5). In fact, these experiments demonstrate a good degree of cross-reactivity between cryptomonad biliproteins and B-phycoerythrin (red algae). C-phycoerythrin (blue-green algae) also could be precipitated by certain antisera to cryptomonad biliproteins but to a lesser degree than B-phycoerythrin (Fig. 4). This result (Fig. 4B) is the first time an antiserum for any cryptomonad is shown to precipitate a C-phycoerythrin. Experiments with antiserum against B-phycoerythrin also show the relationship is valid (Fig. 6) since this antiserum precipitates cryptomonad biliproteins which show lines of partial identity (spurring) homologous to B-phycoerythrin. C-Phycerythrins also are shown to be related to cryptomonad biliproteins (Fig. 6) when both are tested as heterologous antigens against anti-B-phycoerythrin sera. Biliproteins are frequently isolated as mixtures of different aggregates. These aggregates result in multiple precipitin lines on Ouchterlony plates. Antisera to C-phycoerythrin (Fig. 7) and C-phycocyanin (data not shown) have yet to precipitate cryptomonad biliproteins. A very slight reaction is occasionally observed with antiserus against a cryptomonad is tested against C-phycocyanin (Fig. 8). Antiserus against cryptomonad biliproteins failed to show a reaction against red algal allophycocyanin.

Negative results in these experiments sometimes merely indicate the low potency of a particular batch of antiserum or other experimental factors. Absence of immunochemical evidence, therefore, can never be definitive evidence for a lack of relatedness. Sufficient positive results exist to establish that cryptomonad biliproteins are related to blue-green and red algal biliproteins. B-phycoerythrin seems to be especially close to both phycocyanins and phycoerythrins of the cryptomonads. Lines on the Ouchterlony plates suggest cryptomonad biliproteins can be as related to B-phycoerythrin as B-phycoerythrin is to some C-phycoerythrins. A common ancestor is therefore predicted for all algal biliproteins.

In several of these figures results are shown for cryptomonad phycocyanin 612. This is the first time this biliprotein has ever been used in an immunochemical study and it is demonstrated to be related to the other cryptomonad phycocyanin (phycocyanin 645), the two cryptomonad phycoerythrins (phycoerythrins 545 and 566), a red algal biliprotein (B-phycoerythrin), and a phycoerythrin from a blue-green alga (C-phycoerythrin). Its immunochemistry is thus clearly distinctive from C-phycocyanin and R-phycocyanin to which it has some spectral resemblance. In addition to these immunochemical studies on phycocyanin 612, recently its biochemical and spectroscopic properties were evaluated (14, 17). Its chromophore content is unique and differs from that of C-phycocyanin in that its β subunit has a cryptoviolin chromophore in addition to the usual two phycocyanobilins. Its α subunit, however, has the same chromophore content as C-phycocyanin but differs from that found for cryptomonad phycocyanin 645 (15, 18). A common feature in the chromophore compositions of cryptomonad biliproteins is that cryptoviolin is always present (14, 17–19).

It is intriguing that in going from blue-green or red algae to cryptomonad the phycocyanins and phycoerythrins switch from entirely unrelated to almost identical immunochemically. Perhaps this suggests that in the halcyon period when the evolution of the cryptomonads occurred that their photosynthetic unit containing the biliproteins was derived from a single event. Then the subsequent evolvement into six cryptomonad spectral types could occur from the unique happening. The tendencies of cryptomonad biliproteins to most strongly cross-react with the red algal biliprotein also leads to the speculation that biliproteins were incorporated into an ancestral cryptomonad at a fairly advanced time. Bearing in mind the structural similarities between these unicellular motile 'monads' and certain protozoa the last hypothesis cannot be completely denied.

There are two sets of data that support these immunochemical findings; amino acid sequencing of biliproteins and electron microscopy of these organisms. In a few cases, complete amino acid sequences are known for select biliproteins (5, 7, 8, 21–24, 28). Although the entire amino acid sequence of a cryptomonad biliprotein has not yet been completed, partial sequences (11, 20, 25) show regions of strong homology between cryptomonad and noncryptomonad biliproteins. Electron micrographs of thin sections of cryptomonads show the chloroplasts are surrounded by four membranes (Fig. 9), and the chloroplasts of red algae by two membranes (6, 9, 30). The four membrane case can be explained by the proposal that a red alga was entrapped by another eucaryotic organism. An endosymbiotic relationship...
resulted in the red alga degenerating to the cryptomonad chloroplast. The third and fourth membranes around this chloroplast arose from the plasma membranes of the original red alga and the host organism, respectively (6, 9, 30).

The electron microscopy, the amino acid sequencing, and the immunochemistry can be interpreted as suggesting that the relatedness of the biliproteins is a result of endosymbiosis: first of a cyanobacterium becoming the chloroplast of a red alga and then of a red alga becoming the chloroplast of a cryptomonad. Such an hypothesis would be compatible with some of the other nonchloroplast features of the cryptomonad being very different from those of the red alga. This supposition does not accommodate the genesis of Chl c, which is found in the cryptomonads but not in blue-green or red algae.

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