Characterization of a Phycoerythrin without $\alpha$-Subunits from a Unicellular Red Alga*

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We describe here the spectral and biochemical properties of a novel biliprotein belonging to the phycoerythrin family, purified from the phycobilisome of a unicellular red alga, *Rhodella reticulata* strain R6. This biliprotein is assembled from a unique $\beta$-type subunit, chloroplast-encoded, whose hexameric or dodecameric aggregates are stabilized by unusually large linkers (87 and 60 kDa) encoded by the nuclear genome. Although each $\beta$-type subunit bears two phycoerythrobilins and one phycocyanobilin per chain, the linker polypeptides are non-chromophorlated. The apoprotein of the $\beta$-subunit of the *R. reticulata* R6 phycoerythrin is specified by a monocistronic rpeB chloroplast gene that is split into three exons. We discuss the relationships between R6 $\beta$-phycoerythrin and the previously published polypeptide sequences, the structural consequences due to the absence of an $\alpha$-subunit, and its evolutionary implications.

Light is efficiently collected in the prokaryotic cyanobacteria and the eukaryotic Rhodophyta (red algae) by phycobiliproteins (PBP) assembled into macromolecular structures, the phycobilisomes (PBS), found on the outer thylakoidal membranes as extrinsic components (1–10). Phycobiliproteins are oligomeric proteins, built up from two chromophore-bearing polypeptides belonging to two families ($\alpha$ and $\beta$) probably originating from a common ancestor but which apparently diverged early in evolution (11, 12). In PBS, the phycobiliproteins are stabilized by linker polypeptides (L) that are generally colorless. The aggregation states of linker-biliprotein complexes have been typically found as ($\alpha\beta_h$L) or ($\alpha\beta_h$L)$_n$. In cyanobacteria and Rhodophyta, four main classes of biliproteins exist as follows: allophycocyanin (APC), phycocyanin (PC), phycoerythrin (PE), and phycocerythrocyanin (PEC); this last group was found only in cyanobacteria. In other eukaryotes, the unicellular Cryptophyta (or Cryptomonads), resulting from a secondary endosymbiosis (13, 14) between a red algal cell and a colorless eukaryote (15), only one type of biliprotein is synthesized and localized in the thylakoid lumen (8, 16); the $\beta$-subunit is typical of the rhodophyтан $\beta$-PE whereas the $\alpha$-subunit is unlike other PBPs. It is isolated as dimeric ($\alpha\beta$)$_2$ aggregates. Recently, PE has been described at the genomic level in another group of prokaryotic organisms, belonging to the cyanobacterial radiation, the prochlorophyte *Prochlorococcus marinus* (17), but the biochemical characteristics and the intracellular localization of this pigment have not been investigated.

The spectral properties of PBP are due to bilins, linear tetrapyrroles covalently linked to specific cysteinyl residues of the polypeptidic chains by means of one (or less frequently two) thioether bonds. Due to differences in PBP bilin composition, light may be efficiently collected from the blue-green edge to the red part of the visible light spectrum. Moreover, the light absorption properties of the different tetrapyrroles are modulated by molecular interactions with the apoprotein chains in monomers ($\alpha\beta$), in oligomers, and with the specific linker polypeptides.

In prokaryotic organisms (cyanobacteria and Prochlorophyta), the genes for the apoproteins of the two polypeptide chains of a given biliprotein are polycistronic that can be transcribed with genes for specific linker polypeptides as well as for subunits of lyses for chromophore linkage. Cotranscription of the two genes encoding apoproteins is always observed (1, 6, 9, 18). In the eukaryotic Rhodophyta, the PBS apoproteins are encoded by the chloroplast genome, but linkers of the outer PBP (for instance PE linkers, when this pigment present) are nuclear-encoded (19–21). Data from Cryptophyta showed that the $\beta$-subunit of the unique PBP is chloroplast-encoded (22), whereas the $\alpha$-subunit is specified by the nucleus (23) or by the residual nucleus of the first eukaryotic red algal symbiont, the so-called nucleomorph (as proposed in Ref. 9).

During the past 10 years, high resolution crystallographic data have been obtained for representatives of the major groups of phycobiliproteins mentioned above (PC, PEC, PE, and APC). To date, no detailed crystallographic data have been published on cryptophyтан biliproteins. For each of the phycobiliproteins, details of the structural interactions between the $\alpha$- and $\beta$-subunits have been delineated (24–31). Such interactions are essential for assembly of $\alpha\beta$ monomers and of the higher order ($\alpha\beta_h$)$_n$ trimers and ($\alpha\beta_h$)$_h$ hexamers. It has generally been assumed that phycobiliproteins cannot be assembled from only one type ($\alpha$ or $\beta$) of subunit.

We describe here an unusual biliprotein belonging to the PE family from the unicellular red alga *Rhodella reticulata* strain R6. This unique PE, in association with C-PC and APC, forms phycobilisomes similar to the hemidiscoidal type found in cyanobacteria. This PE can be purified as hexamers and dodecamers containing only $\beta$-type subunits, which are stabilized by unusually large, colorless, linker polypeptides (60 and 87 kDa, respectively) specified by the nuclear genome. Moreover, we show that the $\beta$-subunit is encoded by a monocistronic chloroplast gene, interrupted by

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1 The abbreviations used are: PBP, phycobiliprotein; PUB, phycoourbilin; APC, allophycocyanin; PC, phycocyanin; PCC, phycocyanobilin; PE, phycoerythrin; PEB, phycoerythrobilin; PCB, phycocyanobilin; PBS, phycobilisome; L, linker polypeptide; C-PC, C-phycocyanin; PEC, phycocerythrocyanin; kb, kilobase pair; PCR, polymerase chain reaction; nt, nucleotide(s); LiDS-PAGE, lithium dodecylsulphate-polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
two large intronic sequences, one of which is related to a typical group II intron.

**EXPERIMENTAL PROCEDURES**

**Organisms and Culture Conditions**—*R. reticulata* strain R6 (Rhodella grisea (Geitler) Fresnel et al. (32)) was collected in 1983 by C. Billard (Caen University France) from seawater samples from Sarasota Bay, FL, and kindly provided by C. Billard. Two other closely related strains were obtained from the UTEX collection: *R. reticulata* Deason (UTEX LB 2320) and *Dixoniella grisea* (Geitler) Scott et al. (UTEX LB2615) (as *R. reticulata* Deason) for comparison. The two last strains were independent isolates from the U. S.

**Culture Conditions**—All strains were grown on Erdschreiber liquid medium (33) in Erlenmeyer flasks, bubbled with sterile water-saturated air, and exposed to 50 μmol photons m⁻² s⁻¹ of cool fluorescent light under a 16/8 light-dark cycle.

**Phycobiliprotein Purification**—Phycobilisomes were isolated by modifications of the procedure of Yamanaka et al. (34). Typically, exponentially growing cells from 1 liter of culture were collected by centrifugation (4,500 × g, 10 min, Kontron A8.24 rotor), rinsed twice with potassium/sodium phosphate buffer 0.75 m, pH 7, 1 mMEDTA, 1 mM benzamidine (buffer I), resuspended in 40 ml of the same buffer, dispersed with a Teflon homogenizer, and submitted to a French press treatment (AminoCorp) operating at 12,000 p.s.i. Phenylmethylsulfonyl fluoride in solution in isopropyl alcohol (1 mM final concentration) was added to the intermediate blue-violet layer, rapidly mixed, and layered (6 ml) onto a 0.25 to 1.0 discontinuous sucrose gradient in buffer I with 60% (v/v) glycerol (buffer II) and dialyzed overnight at 4 °C. PBS dissociation was achieved as described for *Rhodella violacea* (35) with modifications. The PBS fraction was diluted 1:3 with buffer I and pelleted by centrifugation (210,000 × g, 6 h, 4 °C, Beckman T60 rotor). The pellet was dissolved in 5 mM potassium phosphate buffer, pH 7, containing 10% (v/v) glycerol (buffer II) and dialyzed overnight at 4 °C in the dark. Inhibitors of protease activity (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) were added during dialysis and further purification steps. The lysate was layered onto a linear sucrose density gradient (5–20% sucrose, 20 mM phosphate, pH 7, 10% glycerol) and centrifuged (160,000 × g, 15 h, 4 °C, Beckman T60 rotor). Pigment aggregates banded in the 0.25 to 0.5 M sucrose layers and used for subsequent analysis, whereas small aggregates (<5% total pigments) banded in the 0.25 to 0.5 M sucrose layers.

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**DNA Isolation and Northern Hybridization**—Total RNA was isolated as described previously (40). For the visualization of chromophore-bearing peptides, the gels were stained in situ with Coomassie Blue G-250 (0.1% in 9% acetic acid, 45% methanol) and destained (7.5% acetic acid, 30% methanol) before Southern or library heterologous hybridizations. The DNA sequences of both strands were determined by the dideoxyribonucleotide chain termination method using the Sequenase 2.0 system (U. S. Biochemical Corp.) and [α-32P]dATP with a radioactive counter-stranded DNA primer. RNA isolation and Northern hybridization—Total RNA was isolated as described for cyanobacteria (46). RNA (5 μg) was electrophoresed in 1.2% denaturing agarose gels in HF buffer (0.5 M Heps, 10 mM EDTA, 16% formaldehyde) and transferred to nylon membranes (Hybond-N, Amersham Pharmacia Biotech). Hybridization was at 42 °C with probes as described under “Results.”

**cDNA Cloning and Sequencing**—To sequence cDNA in the junction of two large intronic sequences, one of which is related to a typical group II intron.
between the exons and introns, we performed coupled reverse transcription and PCR amplification: \( R.\ reticulata \) R6 total RNA (5 mg) was used with 0.02 pmol of strict oligonucleotides (see “Results”: \( rpeB \) gene cloning and sequencing), 400 units of reverse transcriptase (Life Technologies, Inc.) in the presence of 20 nmol of each dNTP and 20 units of RNasin (Promega). The reaction was allowed to proceed for 30 min at 37 °C and then inhibited for 5 min at 95 °C. Amplification was performed on the retrotranscription product using a second complementary oligonucleotide as forward primer and the first one as reverse primer. The PCR protocol was the same as above.

5' mRNA Determination—Primer extension was performed for mapping of the 5' terminus of the \( rpe \) mRNA using a strictly complementary 24-mer synthetic oligonucleotide (from nt 1468 to 1441: 5'TCCGTC-CCTTAAACAGGGCCAT3'. 30 μg of total RNA from an \( R.\ reticulata \) R6 exponential culture were used in the primer extension method described by Sambrook et al. (44) modified as in Richaud and Zabulon (47). After ethanol precipitation, the sample was loaded on a sequencing gel together with a sequence standard (pTZ18) in a parallel lane to determine the size of the hybrid.

RESULTS

Phycobilisome and Biliprotein Characterization—After release of \( R.\ reticulata \) R6 phycobilisomes by Triton X-100 treatment of cell lysates, they were recovered from the 0.625 M sucrose layer. They exhibited characteristic absorption features of PEB- and PCB-containing biliproteins (Fig. 1) with a single peak at 562 nm originating from the PEB-containing putative phycocyanin while absorption maxima at 612 and 650 nm (as a shoulder) were attributed to phycocyanin and allophycocyanin, respectively. No shoulder was observed in the 495 nm region, indicating the probable absence of PUB-type chromophores.

The 77 K fluorescence emission spectrum (Fig. 1) exhibited a main peak at 685 nm from the terminal energy acceptors of the phycobilisome and minor peaks at 629 and 651 nm and a 660 nm shoulder. The 651-nm fluorescence peak and the 660 nm shoulder were attributable to uncoupled phycocyanin and allophycocyanin, respectively, but it was not possible before further subtraction of the main biliproteins to determine the origin of the 629-nm fluorescence peak.

We determined the \( R.\ reticulata \) R6 PBS polypeptide composition by LiDS-PAGE (Fig. 2, lane 7). The main biliprotein subunits were in the 15–20-kDa range, and three linkers were 87, 60, and 30 kDa apparent molecular mass, respectively (Fig. 2, lane 7). After zinc acetate treatment, only the biliprotein subunits and the \( L_{CM} \) appeared fluorescent (Fig. 2, lane 8) under UV excitation. The three linker polypeptides (87, 60 and 30 kDa) were clearly non-chromophorylated.

We verified, by labeling experiments of PBS polypeptides in the presence of chloroplastic and cytoplasmic ribosomal translational inhibitors (chloramphenicol and cycloheximide, respectively) as described previously for \( R.\ violacea \) (21), that all the polypeptides of the \( R.\ reticulata \) R6 PBS were chloroplast-encoded. Synthesis of the two PE colorless linkers was blocked by cycloheximide but not by chloramphenicol and therefore are nuclear-encoded (data not shown) as typically is the case for PE linkers in red algae (19–21).

The \( R.\ reticulata \) R6 phycobilisome subcomponents were resolved after gentle dissociation of purified intact particles. Two main fractions were obtained in continuous sucrose gradients. The upper layer (fraction A) contained trimeric aggregates of C-phycocyanin and allophycocyanin and also a minor PE component of low molecular weight (data not shown). The lower part (fraction B) was largely enriched in phycoerythrin but also a minor peak at 629 and a 660 nm (as a shoulder) were attributable to uncoupled phycocyanin and allophycocyanin, respectively. No shoulder was observed in the 495 nm region, indicating the probable absence of PUB-type chromophores.

The different fractions were pelleted separately, and the final step of PC and PE purification was carried out by ultra-centrifugation on linear (5–20%) sucrose density gradients (data not shown). Electrophoretic data confirmed the purity of the \( L_{CM} \)-PC fraction (Fig. 2, lane 1) and in the PE fraction, two non-chromophoric linkers of 87- and 60-kDa apparent molecular mass (Fig. 2, lanes 2–6) were found in addition to the PE subunits in the 20-kDa range. Surprisingly, as previously mentioned, the two PE linkers were clearly non-chromophorylated (Fig. 2, lane 8).

Two distinct complexes of PE (PE I and PE II) were success-
fully resolved by sedimentation through a linear sucrose density gradient (Fig. 4, lower panel). Based on a calibration of the sucrose density gradient with proteins of known molecular weight, PE I was determined to be 184 kDa and PE II to be 300 kDa. The 60-kDa linker (LR<sub>60</sub>) was found in PE I and the 87-kDa linker (LR<sub>87</sub>) in PE II. Consequently, these complexes have designated PE-L<sub>60</sub> and PE-L<sub>87</sub>, respectively.

**Spectral Properties**—The spectral characteristics of the PC and PE pigments were analyzed. The data clearly showed that PC is a C-phycocyanin type, with α- and β-subunits bearing phycocyanobilin chromophores with an absorption maximum at 622 nm and 77 K fluorescence emission at 651 nm (not shown).

PE I and PE II pigments were indistinguishable with respect to their spectral properties with absorption maxima at 562 nm and 615 nm, respectively (Fig. 5). When denatured in acidic 9 M urea, the protein absorption maxima were shifted to 550 and 662 nm (data not shown), demonstrating that the 562-nm peak from phycocyanobilin chromophores, with a second peak at 630 nm (Fig. 5) clearly arises from the phycocyanobilin chromophores with an absorption maximum at 662 nm originating from PCB chromophores, the other peptides were red colored absorbing at 550 nm and so are linked to PEB chromophores (data not shown). No pigmented polypeptides were obtained with 8 M urea.

**Microsequencing and Chemical Cleavage of PE Subunit**—CNBr chemical cleavage was performed on the 9 M urea Bio-Rex fraction. The electrophoretic pattern of cleavage products is shown in Fig. 6. All peptides were chromophore-linked (Fig. 6), and their amino-terminal sequences were determined. The main fragments (6 and 4 kDa) gave the amino sequences MAAS and CLRD and MKASSVA, respectively. The amino-terminal sequence of the minor components (14 and 5 kDa) were identical, that is shown in Fig. 6. All peptides were chromophore-linked (Fig. 6), and their amino-terminal sequences were determined. The main fragments (6 and 4 kDa) gave the amino sequences MAAS and CLRD and MKASSVA, respectively. The amino-terminal sequence of the minor components (14 and 5 kDa) were identical, i.e., CLRD and MKASSVA. We infer that these two peptides were generated by partial cleavage. The amino-terminal sequence was unequivocally of a β-PE type (Fig. 7).

Although the 4-kDa peptide was found to be blue colored, with an absorption maximum at 662 nm originating from PCB chromophores, the other peptides were red colored absorbing at 550 nm and so are linked to PEB chromophores (data not shown).

**Characterization of PE Subunit Composition**—Because of two types of chromophores linked to the subunits of *R. reticulata* R6 PE, we examined the chromophore composition of each putative subunit (α and β). Pure PE I and PE II fractions were separately denatured by acidic 2 M urea and submitted to weak cation exchange chromatography on Bio-Rex 70. In each case, a single colored fraction was eluted with 9 M urea (data not shown). No pigmented polypeptides were obtained with 8 M urea.

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isolated nuclear and plastidic DNA was performed. With probe I (corresponding to the first half of the gene), the only strong signal was with a 2.9-kb plastid DNA fragment (data not shown). This result suggested that a rpeB gene is present as a single copy in the chloroplastic genome of *R. reticulata* R6.

With probe I, we identified positive clones in the EcoRI *R. reticulata* R6 plastid DNA library. One was sequenced using forward and reverse universal primers and internal primers designed upon walking through the insert. The nucleotide and deduced amino acid sequences are shown in Fig. 8. The deduced amino acid sequences revealed a β-type PE amino-terminal sequence and one of the three microsequences obtained from the R6 PE CNBr cleavage products (MAACLDR), but the two coding parts were separated by many stop codons. We concluded that the coding sequences were interrupted by an intron which was confirmed by cDNA sequencing (see below). Furthermore, comparison with other reported β-PE sequences showed that the 3′ region of the *rpeB* gene was missing in this insert.

Because of a unique *PstI* site (positions 1568–1574, Fig. 8) in the insert we prepared a *PstI* library that was screened with another heterologous probe (probe II) corresponding to the central part of the gene. Recombinant plasmids containing a 4.8-kb fragment in the pT7Z *PstI* cloning site were found to contain the 3′-end of the second intron and the *rpeB* 3′-coding sequence. The third microsequence (MKASSVV) obtained from the CNBr cleavage products (see above) is present in the deduced amino acid sequence. Intervening sequences and the splicing sites were identified by synthesizing the cDNA by reverse transcription-PCR with *R. reticulata* R6 total RNA as template, using an oligonucleotide corresponding to the complementary 2800–2820 nucleotide sequence as the reverse primer and the sequence of the 5′ end coding region (nt 490 to 511) as the forward one. The amplification product, 415 nt long, was sequenced (data not shown) and confirmed the junctions between the exon and intron sequences shown in Fig. 8.

A physical map of the two overlapping sequenced clones is shown Fig. 9, and the entire sequence of the *rpeB* gene is presented in Fig. 8.

The first exon is very short and corresponds to the amino-terminal 27 amino acids. The following 0.7-kb intron exhibits the main features of group II introns (48) in which we recognize six putative domains shared by this intron group (Fig. 8). The 5′-end sequence GTAAGC, although unusual, is the exact sequence found in the two *Rhodella* plastid introns known so far, i.e. in the *rpeB* (45) and *pbsA* *R. violacea* genes (47). In addition, the highly conserved nucleotide required for lariat formation, an A located 7 nucleotides upstream from the 3′-splicing site is found (Fig. 8; A+).

Another 1.2-kb intron follows the 180-nt exon 2. It possesses the typical 5′-end of group II introns, GTGGG, but of other characteristic domains only a very weak domain V is present between the 2537 and 2560 nucleotides. None of the specific characteristics for the group I introns were observed.

The organization of the *rpeB* gene in *R. reticulata* R6 is as follows: 5′*-rpeB*(80 nt)-intron(1185 nt)-*rpeB*(183(nt)-intron(1195 nt)-*rpeB*(135 nt)3′.

Thus, only 13% of the nucleotide sequence corresponds to the *rpeB* coding region. We cannot identify other open reading frames in the introns or on the opposite DNA strand. The three exons of *rpeB* exhibit a strong AT bias with GC being 30 to 34%, whereas the first intron shows a smaller value of 20%. Similar values have been observed in the *rpeB* gene from *R. violacea* (45). The second intron in *R. reticulata* R6, although without apparent open reading frame, has a significantly higher percentage of GC (32%). Downstream from the third exon, we observed an inverted repeat of 34 nucleotides able to form a hairpin (nt 2967 to 3003; Fig. 8) followed by a stretch of Ts that would correspond to a rho-independent transcription terminator.

**rpeB** Transcription—The *rpeB* gene transcript size was determined by Northern blot analysis with total RNA using a PCR product (324 base pairs long) as a homologous probe. The two oligonucleotides used as primers were nt 2800 to 2820 and nt 3123 to 3103, respectively. The PCR product obtained with these two primers avoided amplification of the first part (amino acids 91–135, Fig. 7) of the third exon which is very close to βPC. The operon transcript size is 0.7 kb as shown in Fig. 10. For comparison, a Northern blot analysis was also performed with a partial sequence of the *R. reticulata* R6 *cpc* operon (not shown), 239 nt corresponding to the *cpc* β 3′ end, the intergenic part and the 5′ end of *cpc*. The transcript size for *cpc BA* is 1.5 kb, in agreement with the expected size for a transcript covering the entire dicistronic *cpc BA*. In contrast, the *rpe B* tran-
script is significantly shorter.

The 5' end of the mature RNA, determined by primer extension from a strictly complementary oligonucleotide, is at nucleotide A420 (Fig. 8; A420). The sequences TATTAT (nt 406–411) and TTGCGT (nt 381–387), upstream from the transcription start site, are proposed to be the 210 and 235 promoter elements. The GGAG sequence, 7 nt upstream from the coding ATG, is homologous to the Shine-Dalgarno ribosome-binding site.

**DISCUSSION**

We have presented biochemical and molecular evidence of the occurrence of a PE-type pigment assembled from only a b-subunit in the unicellular red alga *R. reticulata* R6. Two other rhodophytan strains (mentioned under “Experimental Procedures”), related to *R. reticulata* R6, isolated independently, have an identical biliprotein composition and synthesize a similar PE pigment. PBS were purified and electron microscope observations indicate that they belong to the hemidiscoidal type. It has been clearly demonstrated that a 95-kDa LCM permits the assembly of three cylinder cores (49, 50), whereas a 72-kDa LCM is associated with two cylinder cores (49, 50) and a 128-kDa LCM corresponds to the so-called four cylinder core (51). The apparent LCM molecular weight (95 kDa) in *R. reticulata* R6 PBS

**FIG. 7.** Comparison of amino acid sequences of b-PE apoproteins from different organisms. Sequences were aligned with the Citi 2 program (Pierre et Marie Curie University, Paris) and optimized by introduction of gaps (—). Amino acid positions are indicated by numbers above the sequences. Dashed lines under the *R. violacea* deduced amino acid sequence indicated peptides used to design synthetic degenerated oligonucleotides. Arrows with top letters above the deduced amino acid sequence noted the putative helical segments. Red algae: *R. r.*, *R. reticulata* R6 (this work); *R. v.*, *R. violacea* (45); *P. c.*, *P. cruentum* (64); *P. b.*, *Polysiphonia* boldii (65); *A. n.*, *Aglaothamnion neglectum* (66). Cyanobacteria: *C. 7601*, *Calothrix* PCC 7601 (46); *S. 6701*, *Synechocystis* PCC 6701 (67); *S. WH 8020 II and I, Synechococcus* sp. WH 8020 (59, 60, 69); *S. WH 7803*, *Synechococcus* 7803 (69); *P. m.*, *Prochlorococcus* marinus (17). Cryptophyta: *C. F.*, *Cryptomonas* F* (22).
FIG. 8. Nucleotide and deduced amino acid sequences of the \textit{rpeB} gene encoding β-PE apoprotein from \textit{R. reticulata} R6. The amino acids in \textbf{bold} correspond to the PE microsequenced fragments. Nucleotide and amino acid positions are given at \textit{right}. The transcription start determined by 5' extension from the synthetic oligonucleotide complementary (nt 1468 to 1441) is noted by an \textit{asterisk}. The proposed −10 and −35 sequences are in \textbf{bold}. " indicates the stop codon, and solid arrows marked the putative terminator. The two homologous oligonucleotides (I and II) used for reverse transcription-PCR and sequencing of the cDNA are indicated by \textit{solid lines}. The group II intron domains are noted by \textit{dashed arrows} (GenBank™ AF114823).
suggests that the R6 PBS is assembled with a tricylindrical core as in many cyanobacteria and some Rhodophyta (52, 53). Preliminary ultrastructural observations (not shown) are in agreement with this hypothesis, but the rod organization remains to be established.

Purification of the major biliproteins after PBS dissociation and DEAE-cellulose chromatography allowed us to purify, in addition to allophycocyanin (APC) and C-phycocyanin (C-PC), a phycocyanin-type pigment. The occurrence of a C-PC has been similarly described in other unicellular or filamentous Rhodophyta as follows: R. violacea (54), Cyanidium caldarium (55), Compsopogon coeruleus (56), and Audouinella species (57). The third pigment, purple-violet in color and responsible for the absorption at 562 nm observed in the phycobilisome fractions from R. reticulata R6, was exhaustively purified. The absorption maxima at 562 and 604 nm originate from PEB and PCB chromophores as shown by the spectral properties obtained after denaturation with acid 9 M urea (absorption maxima at 550 and 662 nm, respectively). No peaks or shoulders were visible in the 495 nm region, indicating that the R. reticulata R6 PE is devoid of PUB. The 77 K fluorescence emission spectrum exhibits a peak at 630 nm that we attribute to the PCB chromophore functioning as a terminal energy acceptor. Surprisingly, zinc-enhanced fluorescence from bilin-linked
chromophores was associated only with a 20-kDa subunit. The 60- and 87-kDa linkers were non-fluorescent, thus appearing devoid of chromophore. Two different linkers could correspond to two different PE forms, but we find they account for two different aggregation states.

The spectral properties of the R. reticulata R6 PE are distinct from other previously described PEs from red algae (9). First, the R. reticulata R6 PE subunits are linked to unusually large colorless polypeptides. In all other rhodophyta PEs studied so far, chromatophoric linkers (the so-called g-type subunits), bearing several PEB and PUB chromophores, were found (58), thus acting as linkers as well as light-harvesting polypeptides (9). In contrast, the PEs found in cyanobacteria are generally stabilized by colorless linker polypeptides, although some exceptions are now known (59, 60). This property shared by the majority of cyanobacterial PE linkers together with the R. reticulata R6 PE linkers may reflect a primitive situation.

Until recently, all PEs were shown to utilize phycoerythrin bilins acting as terminal energy acceptors. However, in the primitive red algal Audouinella and Chantransia strains (57), PEs have been described with a 1:3:1 PCB/PEB/PUB chromophore ratio in which the blue chromophore (PCB) acts as the terminal energy acceptor as well as light-harvesting polypeptides (9). In contrast, the PEs found in cyanobacterias are generally stabilized by colorless linker polypeptides, although some exceptions are now known (59, 60). This property shared by the majority of cyanobacterial PE linkers together with the R. reticulata R6 PE linkers may reflect a primitive situation.

That the α-subunit could be lacking from the R. reticulata R6 PE was totally unexpected. Until now, no exception has been found to a requirement of α- and β-subunits for phycho-biliprotein assembly.

After Edman degradation, only one amino-terminal sequence was identified, MLDAFSKVAVN, which is clearly related to a β-type amino-terminal sequence (17, 22, 45, 46, 59, 60, 64–69; see Fig. 7 for sequence alignment). The five first amino acids (MLDAF) are shared almost all rhodophyta β-PE and β-PC subunit biliproteins, by β-PEC found only in cyanobacteria, and by some cyanobacterial β-PC (12). Serine as the sixth residue is a universal marker for β-PEs, including the prochlorophyta Prochlorococcus PE (17) and for β-PEC. However, β-PEC has a negatively charged amino residue in the tenth position, instead of an aliphatic one in β-PE.

Cloning and sequencing the plastid rpe operon from R. reticulata R6 confirms the occurrence of a unique rpeB gene split into three exons. The occurrence of split genes is not general in plastid rhodophyta genes. However, the R. violacea plastid rpeB gene (45) also has one group II intron (340 nt), and a cis-splicing mechanism produces the mature transcript. The same mechanism seems likely for R. reticulata R6 rpeB although no pre-mRNAs were detected. By primer extension, the transcription start of R. reticulata R6 rpeB gene is localized 71 nt upstream from the ATG initiation codon. Northern analyses using a part of the downstream exon as a probe revealed only one 0.7-kb long transcript, the expected size for the mature transcript between the determined transcription start and the proposed terminator. There is no open reading frame related to a rpeA gene following the rpeB sequence. The size of the mature transcript is consistent with a monocistronic rpe operon and the absence of α-PE in R. reticulata R6.

Analysis of the R. reticulata R6 β-PE amino acid sequence shows it is 84–87% homologous to those of the β-PE subunits of diverse cyanobacteria, red algae, and cryptomonads (Fig. 7; see Refs. 17, 22, 45, 46, 59, 60, and 64–69). Including conservative substitutions of amino acids, the sequence homology with red algal PE (17) and for β-PC is GAACIRDLG (12). The second microsequence MKASSKAFV is not highly conserved.

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bilin is doubly attached at Cys-50 and Cys-61 (58, 63, 71, 72). In cyanobacterial and red algal β-PE sequences, only PEβ has been found at Cys-82. Either PEβ or PUB is found at the other two bilin attachment sites (see Ref. 63). The same bilin attachment sites are found in cryptophytan β-PE, but a greater variety of bilins are found at these sites (16).

The bilins in the R. reticulata R6 β-PE subunit were localized by sequencing and spectral analysis of four fragments (14, 5, 6, and 4 kDa) obtained by CNBr cleavage. The first two (14 and 5 kDa) shared the same amino-terminal sequence indicating that the 14-kDa polypeptide results from incomplete CNBr cleavage. PEβ was associated with 14-, 5-, and 6-kDa CNBr peptides. Its spectral properties show that the 4-kDa fragment represents the COOH terminus of the subunit and that it bears the sole PCB in the R. reticulata R6 β-PE subunit, presumably at the sole cysteine residue in this region, Cys-158 (Fig. 7). The only other instance of a phycocerythrin carrying a PCB chromophore is that of the Audouinella and Chantaransiya sp. phycocerythrin, which also carries the PCB on its β-subunit (57).

Other residues that appeared to be important in chromophore interaction with the polypeptide side chain are present in R. reticulata R6 β PE, for instance Arg-77, Arg-78, Arg-84, Asp-85, and Ala-81 (24, 27, 28, 73, 74). With exception of Arg-78, these residues are maintained in all β-type sequences. In R. reticulata R6 β-PE, there are no tryptophan residues as has been found for all other β sequences. Asn-72, present in most β-PE and in β-PE R6 also, and in some β-PC, has been shown (75) to be post-translationally methylated. This modification is functionally important in the efficiency of energy transfer. A histidine is in this position in Prochlorococcus β-PE indicating possible differences concerning PE in this organism.

Without an α-subunit in R. reticulata R6 phycoerythrin, the question arises whether the β-subunit has a similar three-dimensional structure to those of β-subunits in ‘conventional’ phycobiliproteins. That it probably does is suggested from the high overall homology of its amino acid sequence with that of β-subunits from the other phycobiliproteins (Fig. 7). By modeling from published crystallographic data, we deduce the probable presence of the X, Y, A, B, E, F, G, and H helical segments.

Recall that the bilins of R. reticulata R6 have been located by the method described above (16). The 119-kDa complex contains about 12 bilins, of which 20% are light absorbing and 80% are not. These bilins are contained in two large polypeptides of 45 and 74 kDa (56). The larger polypeptide contains about 80% of the bilins, and the smaller one contains about 20%. The two polypeptides, which are relatively hydrophilic, are circularly permuted and do not form a trimer. The circularly permuted structure is supported by the fact that the two polypeptides are not covalently linked. The circularly permuted structure is also supported by the fact that the two polypeptides are not covalently linked.

The unusual size of the two linkers raises questions about the position of these linkers in the aggregate. In phycocyanin, it is well established that the cavity within the hexameric structure is large enough to bury the larger part of the 30–35-kDa apparent molecular mass linkers (10) leaving an exposed COOH domain in the (αβ)6L aggregate. In phycocerythrin, the structure is similar, but, as determined recently by comparison of crystallographic structure of B- and b-phycocerythrin from Porphyridium, the γ-subunit in B-PE is located inside (αβ)γ hexamers (29) and probably not protruding out of the hexamers.

Conclusion—The main properties of the phycoerythrin from the unicellular red alga R. reticulata R6 are that it has only β chains encoded by a monocistronic plastid gene and that they are organized into two aggregation states (hexameric and dodecameric) stabilized by linker polypeptides significantly larger than linkers usually described for these complexes. Although ββ chain interactions might occur, the two linker polypeptides alternatively might act as α chain substitutes. If the linkers contain peptide domains related to phycobiliprotein sequences, a mosaic origin for these polypeptides, involving fusion of chloroplast and nuclear components, can be considered. However, we cannot yet exclude ββ chain interactions. The cryptophytan biliproteins, with nuclear-encoded α-subunits of unique structure, are thought to be a primitive system (16), which might loosely parallel R. reticulata R6 phycoerythrin. Further analyses at the molecular levels of the two linkers are an essential next step for understanding the structural features of the novel R. reticulata R6 PE.

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