Amino Acid Composition and C-Terminal Residues of Algal Biliproteins

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(Received 19 May 1964)

1. R-phycoerythrin from *Ceramium rubrum* and C-phycoerythrin from *Nostoc muscorum* have been obtained in purified form by fractional crystallization, followed by chromatography and gel-filtration. 2. The amino acid composition of both chromoproteins was determined; 85% of the total weight of both was recovered as amino acids. 3. Alanine was identified as the only C-terminal amino acid of R-phycoerythrin, each molecule of which contained about 12 terminal groups. 4. Serine was identified as the only C-terminal group of C-phycoerythrin.

The algal biliproteins, phycoerythrins and phycocyanins, are chromoproteins whose bile-pigment prosthetic groups are firmly bound to the apoproteins (cf. Lemberg & Legge, 1949; Ó hEocha, 1962). We report some analyses of the protein moieties of R-phycoerythrin and of C-phycoerythrin obtained from the algae *Ceramium rubrum* and *Nostoc muscorum* respectively. In recent years, amino acid analyses of biliproteins from other algal sources have been reported by Fujiwara (1956), Jones & Blinks (1957), Kimmel & Smith (1958) and Berns, Crespi & Katz (1963).

MATERIALS AND METHODS

Isolation of R-phycoerythrin. The littoral marine red alga, *Ceramium rubrum*, collected at Barna, Galway, was our source of R-phycoerythrin. The fresh alga (approx. 2 kg.) was washed and then extracted with distilled water in the dark for several days at 4°. The extract was strained through cheesecloth, clarified further by centrifugation, and then filtered through a short column (2 cm. × 5 cm.) of Super-Cel filter aid.

Purification of R-phycoerythrin. Step 1: precipitation and fractional crystallization. R-phycoerythrin-rich fractions were precipitated from the *Ceramium rubrum* extract by addition of ammonium sulphate to a concentration of 15% (w/v). The resulting precipitate was centrifuged off, dialysed and slowly crystallized by addition of saturated ammonium sulphate in small amounts. After two or three such crystallizations the resulting R-phycoerythrin crystals were free from phycocyanin, as indicated by spectral investigation of their aqueous solution.

Step 2: chromatography on tricalcium phosphate-Super-Cel. The procedure followed was that of Haxo, Ó hEocha & Norris (1955), phosphate buffers being used for the elution of protein fractions from the columns. Protein solutions were dialysed and then made 1% with respect to sodium

chloride before chromatography. Elution was first attempted with 10 mM buffer, pH 6.4; subsequently, the ionic strength of the eluting buffers was increased stepwise until the main biliprotein bands were washed off the columns.

Step 3: gel-filtration on Sephadex G-75. This purification step was carried out on columns (2.5 cm. × 200 cm.) of the cross-linked dextran Sephadex G-75 (Pharmacia, Uppsala), 10 mM-phosphate buffer being used as eluent. This technique (Porath, 1960) was used to free biliprotein solutions from low-molecular-weight contaminants and to desalt crystalline preparations.

Isolation of C-phycoerythrin. It was found necessary to carry out the isolation of this biliprotein under carefully controlled conditions so as to minimize the danger of degradation during isolation, and the method described by Ó Carra (1965) was adopted.

Criteria of purity. (a) Absorption spectra were determined with a Unicam SP.500 spectrophotometer and extinction ratios in the visible and ultraviolet regions were used to estimate purity of the preparations. For R-phycoerythrin the ratio of extinction at 566 mμ relative to that at 280 mμ was used, and for C-phycoerythrin the wavelengths used were 618 and 280 mμ. (b) Starch-gel electrophoresis was carried out at pH 8.5 and 5.0 by the method of Smithies (1955). (c) N-Terminal-group analysis was used as an index of purity of the preparations. This was performed by dinitrophenylation by the method of Sanger (1945), followed by chromatography of DNP-amino acids by the method of Levy (1954).

Amino acid analysis. Before acid hydrolysis, freeze-dried samples of biliproteins were oxidized with performic acid according to the method of Schram, Moore & Bigwood (1954). After evaporation of the performic acid and freeze-drying, the samples were further dried over phosphorus pentoxide in a desiccator. Weighed samples of such oxidized and dried materials were hydrolysed in 6 N-hydrochloric acid at 110 ± 1° in sealed evacuated tubes (Crestfield, Moore & Stein, 1963). Hydrolysis was performed for periods of 20 and 70 hr. For the determination of tryptophan, biliprotein samples were heated with saturated

barium hydroxide solution in an airtight Teflon vial at $110 \pm 1^\circ$ for periods of 10 and 20 hr. All determinations were made according to the method of Spackman, Stein & Moore (1958), with an automatic amino acid analyser (Spinco model 120).

C-Terminal residues. (a) By incubation of the biliproteins with carboxypeptidase-A (Worthington DFP-treated) in 0.2 M-tris buffer, pH 8.4, and identification of the amino acids released after various time-intervals with an amino acid analyser. (b) Hydrazinolysis with anhydrous hydrazine by the modification of Funatsu, Tsugita & Fraenkel-Conrat (1964) of the procedure of Akabori, Ohno & Narita (1952). The free amino acids remaining after the hydrazine treatment were determined by using an amino acid analyser.

RESULTS

Fractional crystallization of the biliproteins resulted in preparations with visible absorption spectra characteristic of R-phycoerythrin and of C-phycoerythrin (Svedberg & Katsurai, 1929). Chromatography of R-phycoerythrin prepared by fractional crystallization showed that it contained colourless protein components that could be separated from it. More than one coloured zone was obtained during chromatography of R-phycoerythrin, the main band being eluted by 0.1 M-phosphate, pH 6.4 (Fig. 1). Starch-gel electrophoresis showed that this main phycoerythrin component was not entirely free from contaminating protein. To further purify this material, it was passed through a column (2.0 cm. $\times$ 200 cm.) of Sephadex G-75, 0.1 M-phosphate, pH 6.4, being used as eluting solvent. Some colourless contaminating material was separated from the main R-phycoerythrin band which emerged at the hold-up volume of the column (Fig. 2).

Purity of preparations. (a) When the ratio of extinction of the main peak in the visible region to

that of the protein peak at 278 m μ was greater than 4.0, the biliproteins were considered highly purified (cf. Svedberg & Katsurai, 1929). R-phycoerythrin purified by steps 1, 2 and 3 had an extinction ratio (566 m μ /278 m μ) 4.37; C-phycoerythrin had an extinction ratio (618 m μ /278 m μ) 4.13. (b) Starch-gel electrophoresis in 0.1 M-veronal buffer, pH 8.5, and in 0.1 M-acetate buffer, pH 5.0, showed that the highly purified preparations migrated as single coloured zones. On staining with nigrosine, no other protein bands were detectable. (c) *N*-Terminal analyses showed that the methods used for purification of R-phycoerythrin effected the disappearance of all *N*-terminal groups except methionine. This has been shown to be the only *N*-terminal residue in this biliprotein (Ó Carra & Ó hEocha, 1962; Ó Carra, 1965).

Amino acid analysis. The recoveries of amino acids from R-phycoerythrin and C-phycoerythrin are presented in Tables 1 and 2 respectively. The values shown are the mean values obtained from three individual analyses for each time of hydrolysis. From these results the recoveries of all amino acids have been calculated. Where hydrolytic destruction or incomplete liberation occurred, extrapolation to zero time or maximal recovery at 70 hr. of hydrolysis yielded the values shown. In Table 3 the results have been calculated to show recoveries of total protein as amino acid residues. For comparative purposes the percentage of total recovered nitrogen due to each amino acid has been calculated. For R-phycoerythrin 84.66% of the hydrolysed biliprotein was recovered as amino acid residues, and for C-phycoerythrin this value was 85.21%. In common with all previous analyses of algal biliproteins, the content of dicarboxylic amino acids was found to be higher than the combined total of

Fig. 1. Chromatography of R-phycoerythrin on tricalcium phosphate-Super-Cel columns (30 cm. $\times$ 1.5 cm.). $\circ$, $E_{275\text{m}\mu}$; $\bullet$, $E_{566\text{m}\mu}$.

Fig. 2. Gel-filtration of R-phycoerythrin on a column (200 cm. $\times$ 2.5 cm.) of Sephadex G-75. The solvent was 0.01 M-phosphate buffer, pH 6.4. $\circ$, $E_{275\text{m}\mu}$; $\bullet$, $E_{566\text{m}\mu}$.

Table 1. *Amino acid recoveries from R-phycoerythrin after acid hydrolysis*

Values are expressed as μ mole of amino acid/0.475 mg. of protein (corrected for 12% moisture, giving a value of 0.418 mg.). The deviations are the average values of the absolute deviations from the mean and their percentage deviations are accordingly calculated. The numbers of determinations were three individual analyses for each time of hydrolysis. Each determination represents a single hydrolysis of an individually hydrolysed protein sample.

Amino acid residue	At 20 hr.		At 70 hr.		Extrapolation to zero time or at maximal recovery
	μ mole	% deviation	μ mole	% deviation	
Asp	0.377	± 0.88	0.371	± 0.53	0.377
Thr	0.180	± 0.55	0.168	± 0.79	0.185
Ser	0.315	± 0.63	0.292	± 0.68	0.324
Glu	0.218	± 0.61	0.216	± 0.62	0.218
Pro	0.091	± 0.73	0.090	± 0.00	0.091
Gly	0.272	± 1.22	0.270	± 0.98	0.272
Ala	0.521	± 0.32	0.520	± 0.38	0.521
Val	0.266	± 2.00	0.268	± 0.75	0.266
Met	0.052	± 2.55	0.053	± 1.25	0.053
Ile	0.164	± 0.40	0.160	± 1.25	0.164
Leu	0.273	± 0.61	0.276	± 0.96	0.273
Tyr	0.115	± 0.87	0.111	± 1.80	0.115
Phe	0.078	± 1.71	0.075	± 0.88	0.078
Lys	0.120	± 0.83	0.119	± 1.68	0.120
His	0.018	± 3.67	0.018	± 3.67	0.018
NH ₃	0.200	± 1.67	0.216	± 1.54	0.194
Arg	0.178	± 1.88	0.178	± 2.60	0.178
CySO ₃ H	0.101	± 1.98	0.099	± 0.67	0.101

Table 2. *Amino acid recoveries from C-phycoerythrin after acid hydrolysis*

Values are expressed as μ mole of amino acid/0.450 mg. of protein (corrected for 10% moisture, giving a value of 0.405 mg.). The deviations are the average values of the absolute deviations from the mean and their percentage deviations are accordingly calculated. The numbers of determinations were three individual analyses for each time of hydrolysis. Each determination represents a single hydrolysis of an individually hydrolysed biliprotein sample.

Amino acid residue	At 20 hr.		At 70 hr.		Extrapolation to zero time or at maximal recovery
	μ mole	% deviation	μ mole	% deviation	
Asp	0.381	± 0.68	0.373	± 0.98	0.381
Thr	0.201	± 0.66	0.193	± 1.03	0.204
Ser	0.231	± 1.42	0.210	± 1.58	0.240
Glu	0.330	± 0.81	0.331	± 0.40	0.331
Pro	0.113	± 1.46	0.110	± 1.20	0.113
Gly	0.288	± 0.46	0.281	± 0.47	0.288
Ala	0.488	± 0.41	0.486	± 0.54	0.488
Val	0.163	± 0.61	0.173	± 1.34	0.173
Met	0.026	± 1.15	0.024	± 1.37	0.026
Ile	0.149	± 1.74	0.159	± 1.67	0.159
Leu	0.275	± 1.30	0.274	± 0.73	0.275
Tyr	0.131	± 0.99	0.126	± 2.11	0.131
Phe	0.086	± 1.11	0.081	± 0.41	0.086
Lys	0.127	± 0.51	0.125	± 0.53	0.127
His	0.031	± 1.06	0.028	± 2.36	0.031
NH ₃	—	—	—	—	—
Arg	0.196	± 1.53	0.192	± 1.73	0.196
CySO ₃ H	0.017	± 2.06	0.014	± 2.36	0.017

Table 3. *Amino acid composition of biliproteins*

Amino acid	Amino acid residue (g./100 g. of protein)		Nitrogen (% of total recovered nitrogen)	
	R-phycoerythrin	C-phycoerythrin	R-phycoerythrin	C-phycoerythrin
Asp	10.38	10.82	9.75	9.90
Thr	4.47	5.09	4.79	5.30
Ser	6.75	5.16	8.38	6.23
Glu	6.73	10.54	5.64	8.60
Pro	2.11	2.71	2.35	2.93
Gly	3.72	4.06	7.04	7.48
Ala	8.86	7.86	13.48	12.68
Val	6.31	4.23	6.88	4.49
Met	1.63	0.84	1.35	0.74
Ile	4.44	4.44	4.24	4.13
Leu	7.39	7.69	7.06	7.14
Tyr	4.49	5.28	2.98	3.40
Phe	2.74	3.13	2.02	2.23
Lys	3.68	4.02	6.21	6.60
His	0.59	1.05	1.35	2.42
NH ₃	0.70*	—	—	—
Arg	6.73	7.66	13.82	15.27
CySO ₃ H	3.64	0.63	2.61	0.45
Total	84.66	85.21	99.95	99.99

* This value is not included in the total.

basic amino acids. Alanine was the amino acid present in greatest amount in both biliproteins. The total elemental sulphur in C-phycoerythrin was accounted for as methionine and cystine sulphur. On the other hand, the elemental sulphur of R-phycoerythrin (1.66%) was greater than that recovered as amino acid sulphur after hydrolysis (1.04%). Analyses of basic hydrolysates indicated the absence of tryptophan in both biliproteins.

C-terminal residues. When portions of the reaction mixture of carboxypeptidase-A and R-phycoerythrin (purified by steps 1, 2 and 3) were withdrawn at various time-intervals and the liberated amino acids determined, the amino acid residue liberated first and in greatest amount was alanine. The biliprotein was found to yield 11.8 alanine residues/mol. of 291000 mol.wt. (Eriksson-Quensel, 1938). Hydrazinolysis confirmed that alanine is the C-terminal residue of R-phycoerythrin. The quantitative value obtained by this method, which usually gives recoveries of 60–75%, was 7.8 alanine residues/mol. of R-phycoerythrin. Carboxypeptidase-A digestion of C-phycoerythrin did not give such clear-cut results but nevertheless indicated that serine was C-terminal. Hydrazinolysis again confirmed this finding. On the basis of mol.wt. 138000 for C-phycoerythrin (Hattori & Fujita, 1959), the quantitative values obtained by these two methods were 1.9 and 1.3–1.8 serine residues/mol. respectively.

DISCUSSION

The prosthetic group accounts for 4–5% by weight of C-phycoerythrin (Clendenning, 1954; Brody & Brody, 1961), and absorption-spectral data indicate that the content of coloured prosthetic groups in R-phycoerythrin is about 7.2% (Ó Carra, 1962). The yields of amino acid residues obtained in our analyses do not account for the remaining mass of either biliprotein. However, R-phycoerythrin from *Ceramium rubrum* contains about 5% of carbohydrate (M. A. Raftery, unpublished work) and, when this value is added to the amino acid and pigment content, 96.8% of the original biliprotein weight is accounted for. The observation that R-phycoerythrin is a glycoprotein was made by Fujiwara (1961), who reported the occurrence of 4.78% of carbohydrate in R-phycoerythrin from *Porphyra tenera*. C-phycoerythrin was not analysed for carbohydrate. The reported amino acid recoveries from biliproteins are: Fujiwara (1956), R-phycoerythrin 63.55%, C-phycoerythrin 67.40%; Kimmel & Smith (1958), R-phycoerythrin 88.76%, C-phycoerythrin 102.24%; Berns *et al.* (1963), C-phycoerythrin 75.54%; this paper, R-phycoerythrin 84.66%, C-phycoerythrin 85.21%.

In common with other workers (Fujiwara, 1956; Kimmel & Smith, 1958) we have failed to account for the total sulphur content of our R-phycoerythrin preparations in terms of the sulphur-containing

amino acids cystine and methionine. If one assumes a recovery of 100% for cystine as cysteic acid, only 62.4% of the total sulphur content (1.66%) of our purest R-phycoerythrin preparation can be accounted for. The presence of tightly bound sulphate-containing polysaccharide may account for the discrepancy in these values (Fujiwara, 1955).

Our results from C-terminal analyses, showing about 12 alanine residues/mol. of R-phycoerythrin, agree closely with quantitative data on N-terminal residues obtained by Ó Carra (1965). Such data indicate the possibility of about 12 sub-units in the R-phycoerythrin molecule. This number is twice that found by Svedberg & Katsurai (1929) by ultracentrifugation; they reported that R-phycoerythrin is partially decomposed into six fragments at pH 11.4. The values for C-phycoerythrin, which are based on a molecular weight approximately half that of R-phycoerythrin, indicate two C-terminal serine residues/mol. This value is about twice that found by Ó Carra (1965) for N-terminal residues in C-phycoerythrin; the basis for the discrepancy between the two values is not known.

This investigation was supported by the Air Force Office of Scientific Research, O.A.R., through the European Office, Aerospace Research, U.S. Air Force [AF-61 (052)-409 and AF63-18], and by the U.S. Public Health Service, Grant no. AM 02691-05 (Cole). The authors thank Professor P. S. Ó Colla for his interest in this problem, Professor R. D. Cole, University of California, for use of an automatic amino acid analyser, Professor E. C. Wassink, Wageningen, for the *Nostoc* culture, Mrs P. Mitchell for growing the *Nostoc* and for assistance in purifying the biliproteins, and Mr R. O'Healy for valuable technical assistance.

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