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MEMBRANE PROTEINS OF *RHODOPSEUDOMONAS SPHEROIDES*

V. ADDITIONAL CHEMICAL CHARACTERIZATION OF A PIGMENT–LIPID-ASSOCIATED PROTEIN ISOLATED FROM CHROMATOPHORES

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SUMMARY

The major protein component, Band 15, of the chromatophores of *Rhodopseudomonas spheroides* is associated with most of the pigments and phospholipids. The primary structure of Band 15 has been further characterized. Cyanogen bromide cleavage produced 3 oligopeptides which were present in equimolar amounts. The sum of the molecular weights of the oligopeptides derived from cyanogen bromide cleavage of Band 15 was 8600. This value compares favorably with the value of 11 000 calculated from the methionine content of the protein. A C-terminal sequence, NH_2...Tyr–Ser–Glu–Glu–(Leu, Ala, Ala, Val, Val, Ala, Ala)–GlyCOOH, is proposed. A tryptic map of the protein has been obtained and the amino acid composition of each tryptic peptide determined.

INTRODUCTION

A major protein component of the chromatophore, isolated from the photosynthetic bacterium, *Rhodopseudomonas spheroides*, is designated Band 15 (Fraker and Kaplan[1]). Band 15 when isolated is associated with most of the pigment and phospholipid found in the chromatophore and therefore must play an important functional and structural role in the chromatophore. The gross chemical composition of Band 15 contained 59% protein, 35% phospholipid, and 6% bacteriochlorophyll[1]. The molecular weight of Band 15 was determined as 9700 by dodecyl sulphate–polyacrylamide gel electrophoresis and 14000 by sedimentation equilibrium; the N-terminus was methionine and the C-terminus was inconclusive but may be glycine[1]. Tryptic digestion gave 10–14 peptides and chymotryptic digestion gave 17 peptides (see Paper IV of this series, Huang and Kaplan[2]).

Because Band 15 represents greater than 50% of the total chromatophore protein and has been shown to be unique to the chromatophore, its ultimate physical and chemical description is essential to a thorough understanding of the structure–function interrelationship of the chromatophore. Further characterization should also add to the knowledge of the structural and functional interrelationship between lipids,

pigments and proteins of the chromatophore and hopefully provide a clearer insight into the general structure of the chromatophore.

METHODS

Growth of organism

Rhodopseudomonas spheroides strain 2.4.1 was grown anaerobically as previously described (Fraker and Kaplan[3]).

Preparation of protein, Band 15

The method of Fraker and Kaplan[3] was used to isolate and purify chromatophores. Preparative dodecyl sulphate–polyacrylamide gel electrophoresis was performed to fractionate chromatophore P_{II} proteins which are soluble in 2-chloroethanol[3]. The isolation and purification of Band 15 was as described by Fraker and Kaplan[1].

Cyanogen bromide cleavage and molecular weight determination of resulting oligopeptide

The associated lipids and pigments of Band 15 were removed by acetone–methanol (7:2, v/v) extraction. The precipitated protein was dissolved in 70% formic acid at 10 mg/ml. Cyanogen bromide was added at 100-fold that of the number methionine residues, and the reaction was allowed to proceed for 20 h at 37 °C in a screw-capped tube[4]. The sample was diluted by addition of distilled water (1:5, v/v) and lyophilized to dryness.

Dodecyl sulphate–polyacrylamide gel electrophoresis was used to determine the molecular weight of the oligopeptides (Swank and Munkres[5]). The gels contained 12.5% acrylamide, 1.25% bisacrylamide, 0.1% dodecyl sulphate, 0.075% *N,N,N′,N′*-tetramethylethylene diamine, 0.07% ammonium persulphate, 8 M urea (ion free), 0.1 M H_3PO_4, and the pH was adjusted to 6.8 with Tris. The electrophoretic buffer contained 0.1% dodecyl sulphate, 0.1 M H_3PO_4, and the pH was adjusted to 6.8 with Tris.

Marker peptides were derived from cyanogen bromide-cleaved cytochrome *c*. The molecular weights of resulting oligopeptides, cytochrome *c* I, II, and III, are 7760, 2780 and 1810, respectively[5]. The digested peptides were dissolved at 2 mg/ml in sample buffer containing 1% dodecyl sulphate, 8 M urea (ion free), 1% mercaptoethanol, 0.1 M H_3PO_4, and the pH was adjusted to 6.8 using Tris. The peptide mixture was heated at 55 °C for 10 min. Approximately 0.1 mg of digested peptides in 0.05 ml sample buffer was layered on the top of the gel. Electrophoresis was performed at 2 mA/tube for 15 h.

The gels were stained in staining solution containing 0.12% Coomassie blue, 4.6% acetic acid, and 25% methanol for 2 h. Destaining was performed by several changes in a solution containing 75 ml acetic acid, 250 ml methanol, and 675 ml water. The gels were scanned according to the method of Marrs and Kaplan[6] at 550 nm using an ISCO Model UA visible light analyzer.

Preparation of ^{3}H-labelled C-terminus of Band 15 (Matsuo et al.[7]).

2 mg of Band 15, associated with lipids and pigments, were placed in a capped conical test tube. 0.1 ml of tritiated water (1 Ci/ml) and 0.2 ml of pyridine was added,

and the solution was shaken vigorously and warmed slightly to enhance the solubility of the protein. 0.05 ml of acetic acid was added to the reaction mixture which was kept under ice. The mixture was incubated at room temperature for 3 h and then, 0.2 ml of pyridine and 0.05 ml of acetic anhydride were added and incubated for another 3 h to enhance ^3H incorporation. The residual tritiated water was removed by distillation.

Carboxypeptidase A digestion

Diisopropylphosphofluoridate-treated carboxypeptidase A (Worthington Biochemical Corp.) was prepared according to Ambler[8]. The concentration of carboxypeptidase A was determined spectrophotometrically using a Zeiss PMQ II Spectrophotometer and a molar absorbance of $8.6 \cdot 10^4$ at 278 nm.

5 mg of Band 15 were dissolved in 1 ml of 0.2 M Tris (pH 7.6) containing 0.5 μmole of norleucine as internal standard. Carboxypeptidase A was added at 5% (w/w) or 2% (mole/mole). The mixture was incubated at 37 °C. 0.2 ml of mixture was removed, and 0.1 ml of acetic acid was added to stop the reaction at intervals of 30 min, 2.75 h. 5 h and 17.75 h. The precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness. The samples were dissolved in 0.6 ml of 0.2 M sodium citrate buffer (pH 2.2), and 0.5 ml was placed on the long column of a Beckman/Spinco Model 120 amino acid analyzer. The acidic and neutral amino acids were determined quantitatively according to Spackman *et al.*[9].

Carboxypeptidases A and B digestion

Diisopropylphosphofluoridate-treated carboxypeptidase B was obtained from Sigma Co. The concentration of carboxypeptidases B was 9.7 mg/ml, and the specific activity was 95 units/mg.

10 mg of Band 15 was dissolved in 1.5 ml of 0.2 M Tris (pH 7.6) containing 1 μmole of norleucine as internal standard. Carboxypeptidase A was added at 5% (w/w) or 2% (mole/mole), and carboxypeptidase B was added at 0.5% (w/w). 0.15 ml of mixture was removed, and 0.1 ml of acetic acid was added to end the reaction at intervals of 15 min, 2 h, 3 h, 4.5 h and 20.5 h. The precipitated protein was removed by centrifugation, and the supernatant was lyophilized to dryness. The samples were dissolved in 1.3 ml of 0.2 M sodium citrate buffer (pH 2.2), and 0.5 ml each was placed on the short and long column of the Beckman/Spinco Model 120 amino acid analyzer.

Amino acid compositions of pyridine–acetic acid–water (pH 6.6)-soluble tryptic peptides

Two-dimensional paper electrophoresis and chromatography was performed on pyridine–acetic acid–water-soluble tryptic digests as described previously[2]. The paper was stained lightly with 0.1% ninhydrin in absolute alcohol. The peptides were eluted from the cut-out areas with distilled water. The eluates were lyophilized to dryness, and the peptides were hydrolyzed in 6 M HCl at 110 °C for 24 h. The amino acids were determined by amino acid analysis.

RESULTS AND DISCUSSION

Cyanogen bromide cleavage

The gel profile of the cyanogen bromide-derived oligopeptides is shown in Fig. 1. Since the amount of Coomassie blue stain absorbed by an oligopeptide is

proportional to the size of the oligopeptide as described by De St. Groth *et al.*[10], the molar ratio of the oligopeptides can be calculated by dividing the band area by the corresponding molecular weight. The results of this estimation indicate that the molar ratio of Band 15 I: Band 15 II: Band 15 III is 1:1:1. Band 15 contains 3 methionine residues and 2 internal methionine residues[1] and should therefore produce 3 oligopeptides after complete cyanogen bromide cleavage. The minimum molecular weight determined from the number of methionine residues is calculated to be 11000.

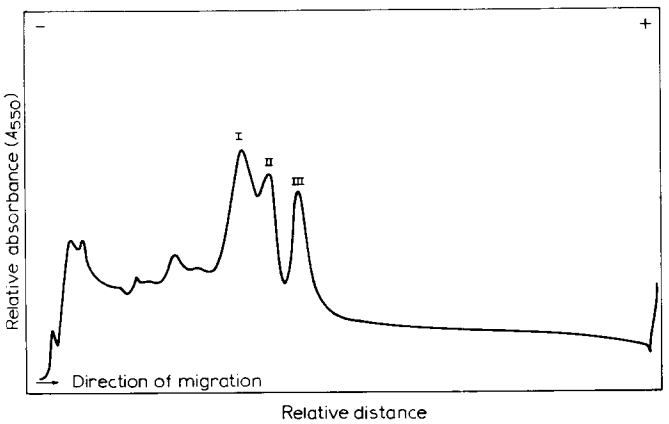

Fig. 1. Dodecyl sulphate–polyacrylamide gel electrophoretic pattern of cyanogen bromide-cleaved Band 15, scanned at 550 nm. The gel consisted of 12.5% acrylamide and 1.25% bisacrylamide. Electrophoresis was carried out at pH 6.8 for 15 h at 2 mA/tube.

To determine the sizes of the cyanogen bromide-cleaved oligopeptides derived from Band 15, cyanogen bromide-digested cytochrome *c* was used as a source of marker peptides. The molecular weights of cytochrome *c* I, II, and III are 7760, 2780 and 1810, respectively. When the mobilities of the oligopeptides are plotted against the log molecular weight using cytochrome *c* I. II and III as standards, Band 15 I, II and III have molecular weights of 4440, 2650 and 1550, respectively (Fig. 2). The sum of the molecular weights is 8600 which is 12% less than the value of 9700 determined by 7% dodecyl sulphate–polyacrylamide gel electrophoresis or 20% less than the value of 11000 calculated as the minimum molecular weight of Band 15. Because the intrinsic charge and conformation of the oligopeptides play a more important role in determining the electrophoretic mobilities of smaller peptides than they do of larger protein (*i.e.* molecular weight above 10000), the molecular weight determination of oligopeptides by urea–dodecyl sulphate–polyacrylamide gel electrophoresis usually has a standard deviation of 18%. However, the procedure still gives us a first approximation on the sizes of the cyanogen bromide-derived oligopeptides. With the successful demonstration of complete cyanogen bromide fragmenting of Band 15 it may now be possible to investigate the relation between chlorophyll and Band 15. We should also point out the presence of larger material on the dodecyl sulphate gels. This undoubtedly consists of partially cleaved Band 15.

Determination of C-terminal sequence

The C-terminal determination of Band 15 by hydrazinolysis was inconclusive[1]. Hydrazinolysis of Band 15 by the resin-catalyzed method gave equimolar quantities of glycine and glutamic acid, while the hydrazine sulphate-catalyzed hydrazinolysis gave only glycine, but the recovery was very low.

A selective ^{3}H-labelling technique was performed to identify the C-terminus of

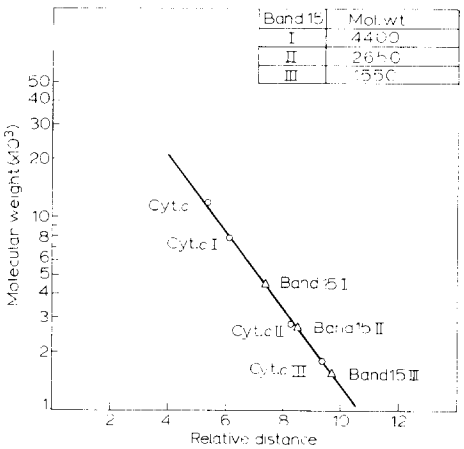

Fig. 2. Determination of molecular weights of cyanogen bromide-cleaved oligopeptides, Band 15 I, Band 15 II, and Band 15 III.

TABLE I

RADIOACTIVITIES OF ^{3}H-LABELLED AMINO ACID FROM PAPER ELECTROPHORESIS AND CHROMATOGRAPHY OF HYDROLYZATES OF BAND 15

0.2 mg of ^{3}H-labelled Band 15 was hydrolyzed in 1 ml of 6 M HCl at 100 °C for 24 h. The hydrolyzates were dried over P_2O_5 and NaOH pellets under vacuum. 0.1 mg of the protein of each hydrolyzate was spotted on either Schleicher and Schuell or Whatman No. 1 paper. Two-dimensional paper electrophoresis followed by chromatography was performed. The electrophoresis was carried out in formic acid–acetic acid–water (10:15:250, by vol.). Ascending chromatography was performed for 2.5 h in a tank equilibrated with *n*-butanol–acetic acid–water (12:3:5, by vol.). The amino acids were detected by spraying with 0.02% ninhydrin in absolute alcohol. The papers were allowed to stand at 60 °C for 20 min or longer until the colour developed. The radioactivity of each amino acid was determined by using a Nuclear Chicago ISOCAP/300 Scintillation spectrometer.

Amino acid	*cpm*	*Amino acid*	*cpm*
Lys	76	Gly	1456
His	8	Ala	0
Arg	40	Val	20
Asx	13	Met	0
Glx	130	Ile	2
Thr	4	Leu	0
Ser	0	Tyr	173
Pro	0	Phe	58

Band 15. The amino acid hydrolysates of C-terminal ^{3}H-labelled Band 15 were separated by two-dimensional paper electrophoresis and chromatography. The radioactivity of each amino acid was determined by scintillation counting. The results shown in Table I clearly demonstrate unambiguously that glycine is the C-terminal amino acid.

Carboxypeptidase A and B hydrolysis

The results from quantitative kinetic studies of carboxypeptidase A digestion of Band 15 are shown in Fig. 3. The recovery of glycine from digestion of Band 15 with carboxypeptidase A is about 0.48 residue per mole of protein assuming a minimum molecular weight of 11000. The sequence degraded by carboxypeptidase A includes 1 residue of glycine, 1 residue of leucine, 4 residues of alanine, 2 residues of glutamic acid, and 2 residues of valine. Because the rate of release of alanine, valine, and leucine per residue is indistinguishable, it is very difficult to place them in sequence. However, the release of the amino acids is proposed to be in the order of Gly > (Ala, Ala, Val, Val, Ala, Ala, Leu) > Glu > Glu > Ser > Phe....

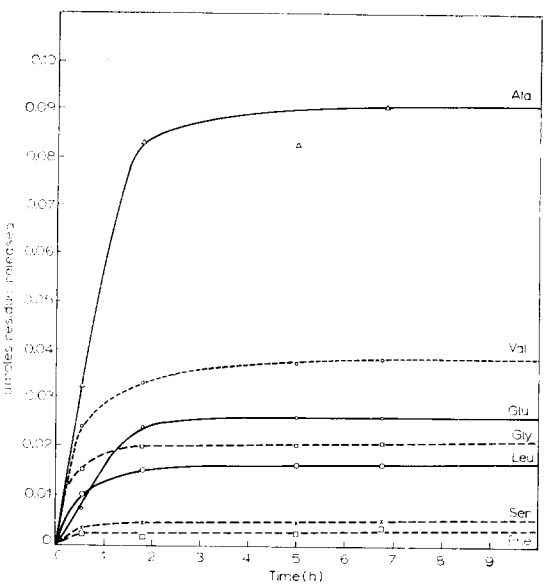

Fig. 3. Rate of release of amino acids from Band 15 by the action of carboxypeptidase A.

The kinetic curves obtained from the digestion of Band 15 with combined carboxypeptidase A and carboxypeptidase B generally confirm the sequence deduced from the digestion with carboxypeptidase A alone (Fig. 4). However, when digested with combined carboxypeptidase A and carboxypeptidase B, serine and tyrosine appear to surpass glycine, and more alanine is released; when digested with carboxypeptidase A alone, they are liberated at a slower rate.

Summarizing all the data at this point, the following C-terminal sequence appears probable: NH_2...Tyr–Ser–Glu–Glu–(Leu, Ala, Ala, Val, Val, Ala, Ala)–GlyCOOH.

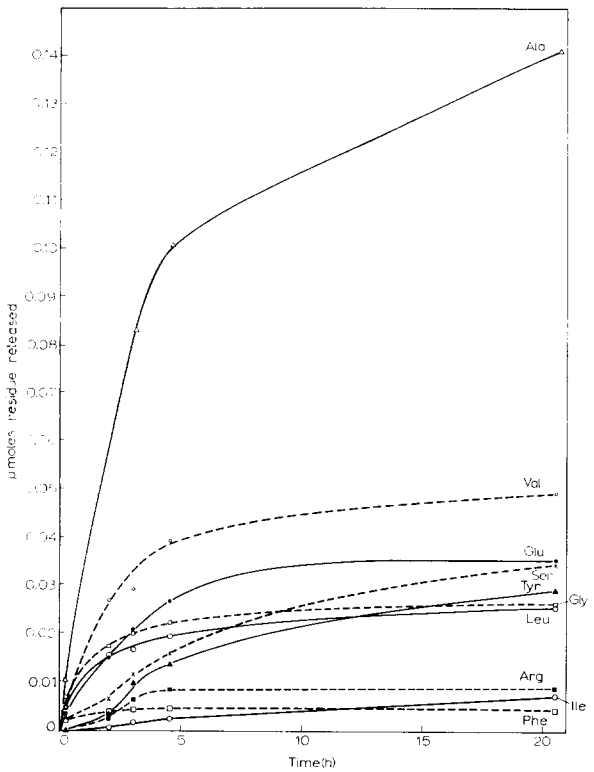

Fig. 4. Rate of release of amino acids from Band 15 by the action of combined carboxypeptidases A and B.

Determination of C-terminal peptide and its amino acid composition

Kinetic experiments with carboxypeptidases give a fairly reliable C-terminal sequence for Band 15. In order to eliminate any misinterpretation, the C-terminal peptide was located and its amino acid composition determined. Tryptic digestion was performed on C-terminal ^{3}H-labelled Band 15, followed by both radioautography of the tryptic fingerprints and scintillation counting of each cut-out peptide (Table II). Both experiments demonstrate that T10 is the C-terminal peptide (see Fig. 7 of ref. 2). In Table III is shown the amino acid composition of the C-terminal peptide, and the C-terminal sequence derived from carboxypeptidases. The correlation between the C-terminal sequence and the amino acid composition of the C-terminal peptide is very good.

Amino acid composition of pyridine–acetic acid–water (pH 6.6)-soluble tryptic peptides

Because of the insolubility of the tryptic peptides of Band 15 at pH 3.1, column chromatography of tryptic peptides on a Beckman ion-exchange resin (sulphonated styrene copolymer spherical resin) was impossible. Preparative paper electrophoresis and chromatography was performed to fractionate the soluble peptide at pH 6.6 in pyridine–acetic acid–water buffer. Approximately 10–14 soluble peptides are shown on the tryptic peptide fingerprint (see Fig. 7 of ref. 2). The amino acid compositions

TABLE II

RADIOACTIVITIES OF ^{3}H-LABELLED PEPTIDE FROM TRYPTIC FINGERPRINT OF BAND 15

Two-dimensional paper electrophoresis and chromatography was performed on a tryptic digest of 1.8 mg of C-terminal ^{3}H-labelled Band 15 (ref. 2). The C-terminal ^{3}H-labelled peptide was determined by spraying the paper with 0.02% ninhydrin in absolute alcohol and heated at 100 °C for 10 min, the developed areas removed and ^{3}H detected by scintillation counting of each peptide.

Peptide	*cpm*	*Peptide*	*cpm*
1	110	5a	34
1a	80	5b	15
1b	0	6	133
2	44	7	38
3	146	8	0
4	301	9	41
5	64	10	23831

TABLE III

THE AMINO ACID COMPOSITION OF THE C-TERMINAL PEPTIDE AND THE C-TERMINAL SEQUENCE DERIVED FROM CARBOXYPEPTIDASE A AND B OF BAND 15

Amino acid	*C-terminal peptide*	*Carboxy-peptidases*
Lys	1	
Asx	1	
Thr	1	Trace
Ser	1	1
Glx	2	2
Pro	1	
Gly	2	1
Ala	4	4
Val	2	2
Met	1	
Ile	1	
Leu	2	1
Tyr	1	1
Phe	1	Trace

of all soluble peptides are shown in Table IV. Each amino acid is expressed as residues per peptide by normalization of either arginine or lysine to one residue. However, some peptides such as T1 and T5 contain 2 lysines; this result may be due to the inability of trypsin to digest some specific lysine bonds.

The tryptic peptide fingerprint of Band 15 is not always reproducible. Peptides T1a, T1b, T5a and T5b do not appear consistently on the tryptic maps. The amino

TABLE IV

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES FROM BAND 15

Results are the molar values of amino acids after 24 h hydrolysis with 6.0 M HCl. Threonine, serine, and tyrosine were not corrected for hydrolytic losses. Figures in parentheses refer to the theoretical values based on analysis.

Amino acid	*T1*	*T1a*	*T1b*	*T2*	*T3*	*T4*	*T5*	*T5a*	*T5b*	*T6*	*T7*	*T8*	*T9*	*T10*	*Total**	*Calcd***
Lys	2.0(2)	1.0(1)	1.0(1)	2.0(2)		1.0(1)	1.0(1)	1.0(1)			1.0(1)			1.0(1)	8	5
His							0.3(0)								0	0
Arg					1.0(1)					1.0(1)		1.0(1)	1.0(1)		4	4
Asx	2.4(2)	2.0(2)		0.9(1)	1.3(1)	2.1(2)	1.0(1)				1.0(1)		1.4(1)	1.2(1)	10	6
Thr	1.3(1)	0.9(1)				1.1(1)	1.3(1)			1.0(1)				1.0(1)	5	6
Ser				1.7(2)		0.7(1)	1.3(1)				0.8(1)			1.0(1)	6	8
Glx				0.7(1)	0.7(1)	2.2(2)	3.9(4)		3.0(3)	1.0(1)				2.0(2)	11	8
Pro	(1)				(1)	(1)	(1)							0.6(1)	5	5
Gly	1.7(2)	0.8(1)	0.7(1)	1.0(1)		1.8(2)	1.7(2)	0.5(1)	0.5(1)	1.0(1)	1.2(1)			2.2(2)	11	8
Ala	1.0(1)	0.4(1)		0.7(1)	0.9(1)	2.0(2)	4.4(5)		4.6(5)					4.1(4)	14	14
Val				0.7(1)		1.6(2)	4.5(5)		4.7(5)					1.6(2)	10	12
Met	1.3(1)	0.8(1)		1.4(1)										0.6(1)	3	3
Ile						0.8(1)	0.7(1)			0.8(1)				0.6(1)	4	6
Leu	0.4(1)	0.5(1)		0.5(1)		1.6(2)	1.7(2)			1.8(2)				1.5(2)	10	12
Tyr						0.1(0)	0.4(1)	0.9(1)					0.8(1)	0.7(1)	3	3
Phe						0.3(1)	0.3(1)	0.7(1)						0.5(1)	3	5
No. of residues	11	8	2	11	5	18	26	4	14	7	4	1	3	21	107	105

* T1a, T1b, T5a, and T5b were not included in this summation.

** Molecular weight of 11000 was used.

acid composition data of Peptides T1a, T1b, T5a and T5b indicate that T1a and T1b may be derived from T1, and T5a and T5b may be derived from T5.

Generally speaking, there is a correlation between the sum of each amino acid residue of all the peptides and the expected amino acid residue calculated from the amino acid composition data of Band 15 using the molecular weight of 11000 for Band 15. Lysine, aspartic, and glycine residues are higher than the expected value, this may be due to the overlapping of some lysine-containing peptides which are counted more than once. On the other hand, serine, alanine, isoleucine, leucine and phenylalanine residues are lower than the expected value; this may be explained by the presence of insoluble material at the origin. The recovery of the insoluble material from the origin of the tryptic peptide fingerprints is very low, so there is as yet no definite conclusion.

Finally, we would like to consider something of the three-dimensional structure of Band 15 and its possible structural relationships within the chromatophores of *R. spheroides*.

According to Fisher[11], $P = V_{\text{polar}}/V_{\text{non-polar}}$, where P is the polarity ratio, V_{polar} is the volume of the polar amino acids and $V_{\text{non-polar}}$ is the volume of non-polar amino acids. The polarity ratio (P) of Band 15 was calculated to be 0.61 from the amino acid composition data (Fraker and Kaplan[3]).

When the molecular weight of 14000 (determined by sedimentation equilibrium) und $\bar{v}$ of 0.78 ml/g (calculated from the amino acid composition[3]) of Band 15 were ased, the total volume (V_t) of the protein portion of Band 15 was calculated to be $1.8 \cdot 10^{-20}$ cm^3. Using equation $V = 4/3\pi r^3$, the radius (r) for a perfect sphere was 16.3 Å.

According to Fisher[11] $P_s = [r^3/(r-d)^3] - 1$ where P_s is the polarity ratio for a perfect sphere, d is the thickness of the polar monolayer which is assumed to be 4 Å, r is the radius. P_s was calculated to be 1.37.

The value of $P = 0.61$ is very much smaller than the value of $P_s = 1.37$, suggesting there are not enough polar amino acids to cover non polar residues completely, therefore, some of the hydrophobic groups must also be exposed. In this case, the protein would be expected to interact with itself and aggregate. Using the data presented by Fisher[11], the minimum size of this aggregate would be expected to 4–6 monomer units. What role the phospholipid and chlorophyll play in such a structure remains to be determined. Experimentally, we know that the biosynthesis of the chromatophore *in vivo* appears to take place *via* the intercalation of relatively large subunits throughout the entire length of the lamellar structure, (Kaplan, S. unpublished experiments).

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