Isolation and Chemical Properties of Two *c*-Type Cytochromes of *Rhodospirillum molischianum**

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ABSTRACT: Two *c*-type cytochromes were isolated in good yields and obtained in pure form from cells of *Rhodospirillum molischianum*. One of these proteins contains 122 amino acid residues and appears to belong to the class of cytochromes c_2, present in most photosynthetic bacteria; the polypeptide chain of the other protein, however, is only 93 residues long. The sequences of the first five residues, starting from the N termini, are established by Edman degradation and found to be identical in both heme proteins: H-Ala-Asp-Ala-Pro-Pro. Structural similarities between these two cytochromes are also indicated from their C-terminal sequences and from the patterns of their tryptic digests obtained both by column chromatography and by peptide mapping.

In the course of comparative sequence studies on *c*-type cytochromes of photosynthetic bacteria, extensive sequence homology was revealed between cytochrome c_2 of the facultative photoanaerobe *Rhodospirillum rubrum* and cytochrome *c* of eukaryotic organisms (Sletten *et al.*, 1968; Dus *et al.*, 1968). Considering the evolutionary significance of this finding, it was tempting to extend these studies to the photoheterotrophic and strictly anaerobic *Rhodospirillum molischianum*, which can be obtained in mass cultures using techniques described previously (Kamen *et al.*, 1963). From this research it was soon learned that the same cell of *R. molischianum* synthesized at least two different *c*-type cytochromes (Dus *et al.*, 1969) called cytochrome c_2 and small *c*-type cytochrome.

The present paper describes the isolation and the chemical properties of these two cytochromes; their physicochemical properties are reported in the accompanying paper (Flatmark *et al.*, 1970).

Materials

Ampholyte solutions for isoelectric fractionation by electrofocusing were purchased from LKB-Produkter AB, Stockholm, Sweden. All chemicals used were reagent grade, with the exception of *N*-bromosuccinimide which was twice recrystallized from water before use. Solvents and reagents used for the Edman degradation were carefully purified according to the recommendations of Edman and Sjöquist (1956).

Enzymes for partial digestion (trypsin, leucine aminopeptidase, and carboxypeptidase A) were highly purified preparations from Worthington Biochemical Corp. Pronase was purchased from Calbiochem. Horse heart cytochrome *c*, type III, was obtained from Sigma Chemical Co.

Microorganism, Growth Medium, and Cultivation. Cells of *R. molischianum* (No. 14031 ATCC) were grown in uniformly illuminated (average of about 100 ft candles using 60-W GE Showcase tubular lamps) 1-l. prescription bottles at 35°. A modified Hutner medium was used with succinate and malate as organic carbon sources (Kamen *et al.*, 1963). In separate experiments, batches of cells were collected with a Sharples centrifuge after 1-, 2-, 4-, and 5-days growth, respectively. Under these conditions, yields averaged from 6.5 g wet paste/l. for 1 day, to 9 g wet paste/l. for 5 days.

Partial Purification of c-Type Cytochromes. The extraction of the soluble heme proteins and the initial purification steps were essentially the same as described for cytochrome c_2 of *R. rubrum* (Kamen *et al.*, 1963). The cellular debris resulting from sonication at pH 7.0 (about 5 min, 10°) was removed by centrifugation (40,000*g*, 4°, 20 min). The heme proteins were isolated from the supernatant by ammonium sulfate fractionation. A first precipitate obtained after 40% saturation at pH 7.0 was discarded. Further addition of ammonium sulfate to a final concentration of 95% saturation produced a second precipitate which contained most of the extracted heme protein. This precipitate was redissolved in 0.1 M phosphate buffer (pH 7.0) and desalted on a column of Sephadex G-25. Subsequent column chromatography on DEAE-cellulose, using 0.001 M Tris-HCl buffer (pH 7.0) permitted the cytochromes to pass through, while particles and a large amount of contaminating protein were retained on the column. The cytochrome fraction was then adsorbed onto a column of CM-cellulose, equilibrated with the same buffer as was used in the DEAE-cellulose step, and the cytochromes were eluted with 0.01 M Tris-HCl buffer (pH 7.5). The eluate was dialyzed against dilute ammonia and lyophilized.

Under these conditions, 1 kg of wet cell paste yielded *ca.* 80 mg of *c*-type cytochromes.

Final Purification Steps. The partially purified cytochrome

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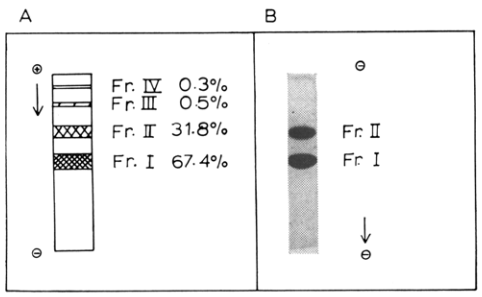

FIGURE 1: Disc electrophoresis of *c*-type cytochromes of *R. molischianum*. The schematic pattern in part A refers to separations by preparative gel electrophoresis of the mixtures of the cytochromes obtained after several purification steps. The percentages of individual components in the mixture are based on the absorbance at 415 nm of the eluted cytochromes after reduction with sodium dithionite. The photograph in part B represents the resolution of an artificial mixture of fractions I and II in an analytical run. The gel was stained with Amido-Schwarz 10B and destained electrolytically.

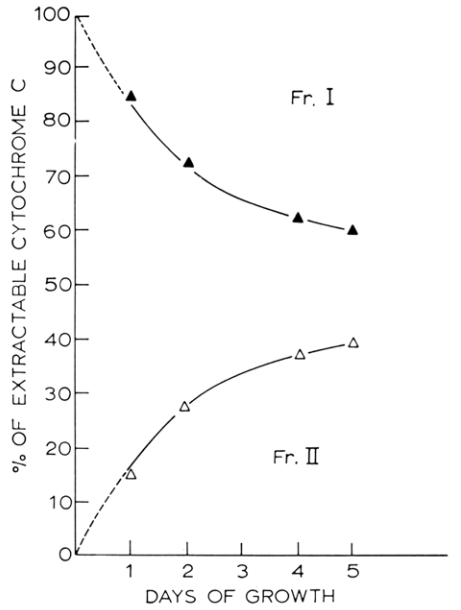

FIGURE 2: Variations in amounts of extractable *c*-type cytochromes. The contributions of cytochrome c_2 (fraction II) and small *c*-type cytochrome (fraction I) to the total amount of *c*-type cytochromes extractable after a given period of growth are based on the optical density at 415 nm of the reduced components separated by disc electrophoresis.

preparation was then subjected to electrofocusing in a combined density (sucrose 0–50%) and pH (3–10) gradient (Vesterberg and Svensson, 1966). One rather broad cytochrome band was formed close to the cathode indicating strongly basic pI's of all the components of the cytochrome fraction. Because of the limitations of the pH range of the commercially available ampholine solutions (pH 3–10) no separation into individual cytochromes was achieved by this technique. The removal of uncolored proteins, however, gave material of much higher purity than was applied. The capacity of the column was 30 mg of cytochrome. After draining the column, sucrose and ampholytes were removed from the cytochromes by dialysis against 1 mM ammonia (three changes in about 6 hr), followed by electrodialysis (2–3 hr) against 1 mM phosphate buffer (pH 7.0).

The electrofocusing step resulted in a substantial enrichment of the cytochrome preparations before the final separation of the individual cytochromes by preparative disc electrophoresis on polyacrylamide gel (Flatmark, 1964). The internal diameter of the glass tube was 9 mm, and the electrophoresis was run at constant current of 7.5 mA. A maximum of 3 mg of cytochromes *c* was applied in each run. The runs were terminated after 2 hr and the separated components were removed from the minced gel sections by repeated extractions at 4° with 0.04 M ammonium phosphate buffer (pH 6.9). Each fraction eluted from the gel was dialyzed against 1 mM ammonia and lyophilized. Samples of this material were rerun in an analytical gel with the same technique to ensure that no contamination had occurred during the cutting of the gel.

Amino Acid Analysis. Before hydrolysis, each cytochrome preparation was redissolved and passed through a column of Sephadex G-10 (1 × 10 cm) equilibrated with distilled water. Protein samples (about 0.5 mg) were then hydrolyzed with 5.9 N HCl in sealed glass tubes for 24 and 48 hr at 105–110°. Decomposition of tyrosine and methionine during hydrolysis was almost completely prevented by the addition of thioglycolic acid (final concentration 0.05%, v/v) to the HCl. Amino acid analyses were performed on a modified Beckman-Spinco Model 120 amino acid analyzer, using the stepwise four-buffer elution program of Dus *et al.* (1966, 1967c). The contents of glutamine and asparagine were determined from enzymic hydrolysates (Sletten *et al.*, 1968) applying the chromatographic procedure of Benson *et al.* (1967). Cysteine and cystine were measured as cysteic acid after performic acid oxidation and subsequent acid hydrolysis (24 hr) of the protein sample (Hirs, 1956). Tryptophan was determined by two independent techniques. Differential spectrophotometric titration with *N*-bromosuccinimide (Patschornik *et al.*, 1958) was carried out in 0.2 M sodium acetate buffer (pH 4.1) using as titrant a 1.0×10^{-4} M solution of *N*-bromosuccinimide in the same solvent. In addition, amino acid analysis of the enzymic hydrolysate was performed using the four-buffer elution program (Dus *et al.*, 1966, 1967c). Under these conditions, tryptophan emerged in the fourth buffer region, well separated from both ammonia and arginine.

End-group analyses, including dinitrophenylation (Dus *et al.*, 1967b), stepwise Edman degradation (Sletten *et al.*, 1968; Doolittle, 1965; Edman and Begg, 1967) and digestion with carboxypeptidase A (Dus *et al.*, 1962) were performed on highly purified preparations of the native protein as described previously, except that quantitation of the free amino acids released by carboxypeptidase was effected on the automatic amino acid analyzer instead of using dinitrophenylation. The aliquots withdrawn from the digest at certain time intervals were acidified immediately with a few drops of 2 N HCl. After dilution with 1 ml of 0.2 N citrate buffer (pH 2.2), the precipitate was removed by centrifugation, and the supernatant was applied directly to the amino acid analyzer column. The presence of amides was investigated using the Li buffer scheme of Benson *et al.* (1967).

Tryptic Digests. The native cytochromes were digested

TABLE I: Amino Acid Compositions.

	Small *c*-Type Cytochrome[a]					Cytochrome c_2[a]				
	1	2	3	4	5	1	2	3	4	5
Cysteic acid			1.9		2			2.1		2
Aspartic acid	9.9	9.7	10.8[b]	4.6	6	12.1	11.8	13.5[b]	7.3	8
Methionine sulfone			2.8					1.0		
Threonine	3.7	3.8	4.5[b]	4.3	4	7.8	7.5	8.4[b]	10.1	8
Serine	3.5	3.1	3.9	4.6	5	5.4	4.8	6.0	7.0	7
Asparagine				4.1	4				3.5	4
Glutamic acid	1.9	2.2	1.7	0.7	1	6.2	6.0	6.5	3.0	5
Glutamine				0.6	1				1.1	1
Proline	8.0	8.1	8.6[b]	2.6	8	8.7	8.9	9.8[b]	3.1	9
Glycine	9.8	10.2	10.9[b]	8.1	10	12.7	12.9	14.6[b]	11.0	13
Alanine	15.1	14.8	15.8[b]	11.2	15	14.7	14.9	17.0[b]	12.4	15
Valine	4.3	4.9	5.4[b]	5.1	5	7.8	8.1	9.3[b]	7.6	8
Methionine	2.9	2.5		1.3	3	1.0	0.7		0.5	1
Isoleucine	3.5	4.0	4.3[b]	3.4	4	3.7	3.9	4.7[b]	3.3	4
Leucine	6.1	6.2	6.7[b]	4.1	6	8.7	9.1	9.8[b]	6.4	9
Tyrosine	4.6	4.1	2.4[b]	5.4	5	3.8	3.4	2.1[b]	4.9	4
Phenylalanine	2.2	2.1	2.4	2.0	2	4.1	4.0	4.1	2.9	4
Histidine	2.0	2.0	2.0	2.0	2	2.0	2.0	2.0	2.0	2
Lysine	9.2	9.0	9.2[b]	8.7	9	13.5	13.9	15.2[b]	11.1	14
Tryptophan					0				1.1	1
Arginine	0.8	0.7	1.2	1.0	1	2.3	2.4	1.9	2.7	3
					93					122
Number of heme groups					1					1
Molecular weight					10,221					13,443

[a] (1) Acid hydrolysis for 24 hr. (2) Acid hydrolysis for 48 hr. (3) Acid hydrolysis for 24 hr after performic acid oxidation. (4) Complete enzymic hydrolysis. (5) Residues to nearest integer. The values are based on the occurrence of two histidyl residues in both proteins and refer to the average recovery of residues from a series of hydrolyses of each type. [b] Values less reliable after performic acid oxidation due to possible contributions of breakdown products from heme and labile amino acids.

with trypsin at 37° for 5 hr, using a molar enzyme:substrate ratio of 1:30. The enzyme preparation had been treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone to inactivate traces of contaminating chyomotrypsin (Kostka and Carpenter, 1964). The digestions were carried out in 2% sodium bicarbonate buffer (pH 8.5) for subsequent column separation of the peptides, or in dilute ammonia (pH 8.5–8.7) for the preparation of peptide maps.

The comparative study of the tryptic peptides by separation on ion-exchange resin followed the procedure of Jones (1964). The column effluents were monitored with a Gilford spectrophotometer, Model 2000, attached to a Beckman-Spinco Model 120 amino acid analyzer (Dus *et al.*, 1965). A column of spherical research resin (1.3 × 28.0 cm, PA-35) and a linear gradient between 0.2 M pyridine-acetate (pH 3.1) and 2.0 M pyridine-acetate (pH 5.0) were used, maintaining a flow rate of 30 ml/hr at 55°.

Spectrophotometric Determinations. Absorption spectra were recorded on a Cary recording spectrophotometer, Model 14. The cytochromes were dissolved in 0.04 M ammonium phosphate buffer (pH 6.9). The fully reduced and oxidized forms of cytochromes c_2 were easily obtained by addition of minimal amounts of dithionite or ferricyanide, respectively. To ensure complete oxidation of the small *c*-type cytochrome, however, it became necessary to use a 20-fold excess of ferricyanide. The absorbance of this solution was measured against that of a blank containing the same amount of ferricyanide. The pyridine hemochromogens were prepared according to the recommendations of Gallagher and Elliott (1965) using a mixture of 0.5 ml of 1 N NaOH and 1.5 ml of 25% pyridine.

Results

Disc electrophoresis on polyacrylamide gel (basic system) revealed the presence of two major and two minor fractions of cytochrome *c*, which will be designated as fraction I–IV in order of decreasing positive charge (Figure 1A). Preparative disc electrophoresis gave their relative percentages as: fraction I 67.4%, fraction II 31.8%, fraction III 0.5%, and fraction IV 0.3% in cells grown for 3 days. Each subfraction appeared quite homogeneous and maintained its relative mobility when rerun on disc electrophoresis (Figure 1B).

A preliminary series of growth experiments (Figure 2) revealed a gradual increase in the percentage of fraction II.

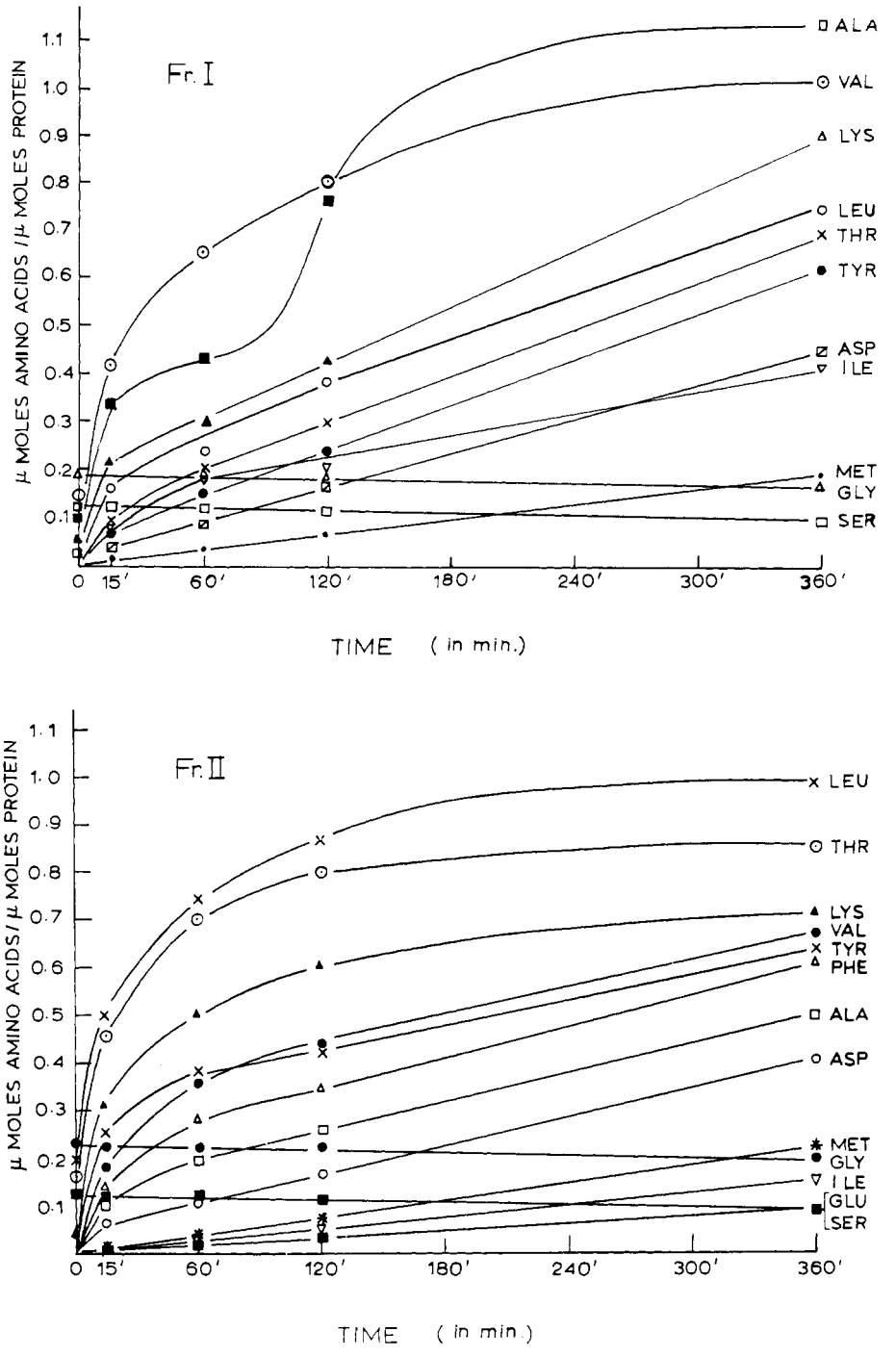

FIGURE 3: Digestion with carboxypeptidase A. Identical aliquots were removed from the digests after 15, 60, 120, and 360 min. After acidification and removal of insoluble material, the amino acid composition of the aliquots was determined by quantitative amino acid analysis on a Beckman-Spinco, Model 120, amino acid analyzer. These values were corrected for contributions of amino acids resulting from self-digestion of the enzyme during the corresponding time intervals.

Following 5 days of growth this fraction comprised about 40% of the total amount of low molecular weight *c*-type cytochromes.

A mixture containing equal amounts of pure cytochrome c_2 (fraction II) and small *c*-type cytochrome (fraction I) of *R. molischianum* and native bovine heart cytochrome *c* (Flatmark, 1964) traveled as a single band when subjected to molecular sieve chromatography on a column of Sephadex G-75, equilibrated with 0.04 M ammonium phosphate buffer (pH 6.9). The K_d value obtained was characteristic of the monomeric form of bovine heart cytochrome *c*.

Although the two minor components, fraction III and fraction IV, may be artifacts, it is now well established (see below) that the same cell of *R. molischianum* synthesizes

TABLE II: End-Group Analyses.[a]

	Small *c*-Type Cytochrome	Cytochrome c_2
N terminus		
1. Sanger's 1-fluoro-2,4-dinitrobenzene procedure	Ala (92%)	Ala (86%)
2. Edman degradation	H-Ala-Asp-Ala-Pro-Pro	H-Ala-Asp-Ala-Pro-Pro
C terminus		
1. Digestion with carboxypeptidase A	(Ile, Asp, Ala, Tyr, Lys, Thr, Leu, Lys)-Ala-Val-OH	(Asp, Ala, Phe, Tyr, Val)-Lys-Thr-Leu-OH

[a] All procedures were carried out on the heme proteins without prior denaturation or modification.

at least two distinct molecular forms of cytochrome *c* monomer, namely, cytochrome c_2 and the small *c*-type cytochrome.

Both proteins show the characteristic spectroscopic properties of normal *c*-type cytochromes. The maxima of all absorption bands in the visible region are identical for fractions I and II (Flatmark *et al.*, 1970). The millimolar extinction coefficients of the reduced α bands, ϵ_{550}, are 29.76 and 29.15 for the cytochrome c_2 and the small *c*-type cytochrome, respectively; the millimolar extinction coefficients, ϵ_{550}, of the corresponding pyridine hemochromogens were found to be 30.3 and 29.8. The values are based on quantitative amino acid analyses of identical aliquots.

The amino acid compositions of cytochrome c_2 and of the small *c*-type cytochrome are given in Table I. By adding the weight of one mesoheme IX to the weight of the total number of amino acid residues, a formula weight of 13,443 was obtained for the cytochrome c_2. A corresponding value of 10,221 was calculated for the small *c*-type cytochrome.

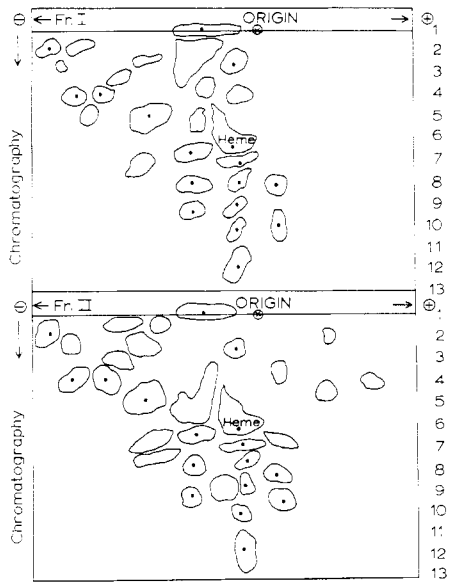

FIGURE 4: Peptide maps of the tryptic digests. After application of 0.1 μmole of digest to full sheets of Whatman No. 3HR, electrophoresis was performed in pyridine-acetate buffer (pH 4.7) in the first dimension using 2 kV for 80 min. Subsequent descending chromatography was carried out at right angles using the solvent system: butan-1-ol–acetic acid–water (4:1:5) at 20° for 16 hr. Spots in similar positions are marked by a center dot.

Differential spectrophotometric titration of cytochrome c_2 with *N*-bromosuccinimide yielded a maximal increase in extinction at 280 nm of the untreated *vs.* the treated aliquot, after the addition of reagent, in amounts corresponding to 0.98 residue of tryptophan. This yield is based on the expected consumption of 3 moles of *N*-bromosuccinimide per mole of tryptophan. In parallel experiments, only 0.05 residue of tryptophan was found in the small *c*-type cytochrome (fraction I) while horse heart cytochrome *c*, employed as a reference substance, gave a value of 0.93 residue of tryptophan.

Each protein consists of a single polypeptide chain with alanine in the amino-terminal position. No trace amounts of other amino acids were found by dinitrophenylation, confirming the purity of the preparations and permitting an independent check of the molecular weights obtained by amino acid analysis.

The results of stepwise Edman degradation and of the release of amino acids by carboxypeptidase A are summarized in Table II. From the time course of the latter reaction, it can be concluded that two alanyl residues are located close to the carboxyl terminus in the small *c*-type cytochrome with one of them taking the penultimate position (Figure 3A). In both cytochromes (Figure 3A,B), small amounts of glycine and serine were found after digestion with carboxypeptidase A and these amounts declined with incubation time. Since no amino acids other than alanine were encountered during end-group analyses at the amino termini these traces could not result from contamination of the protein with free amino acids. Somewhat unusual curves for release of isoleucine in fraction I, and of tyrosine in fraction II, were also obtained. At present the meaning of these results is obscure.

The peptide maps of the tryptic digests of fractions I and II are compared in Figure 4. A large number of ninhydrin-positive spots of various intensities was readily recognizable by visual inspection. This number was far in excess of the total number of basic residues. Since chymotryptic activity had been eliminated by treatment of the trypsin preparation with L-1-tosylamido-2-phenylethyl chloromethyl ketone, this finding was unexpected. A significantly smaller number of constituent peaks was found in the same digest after separation by column chromatography on cation-exchange resin

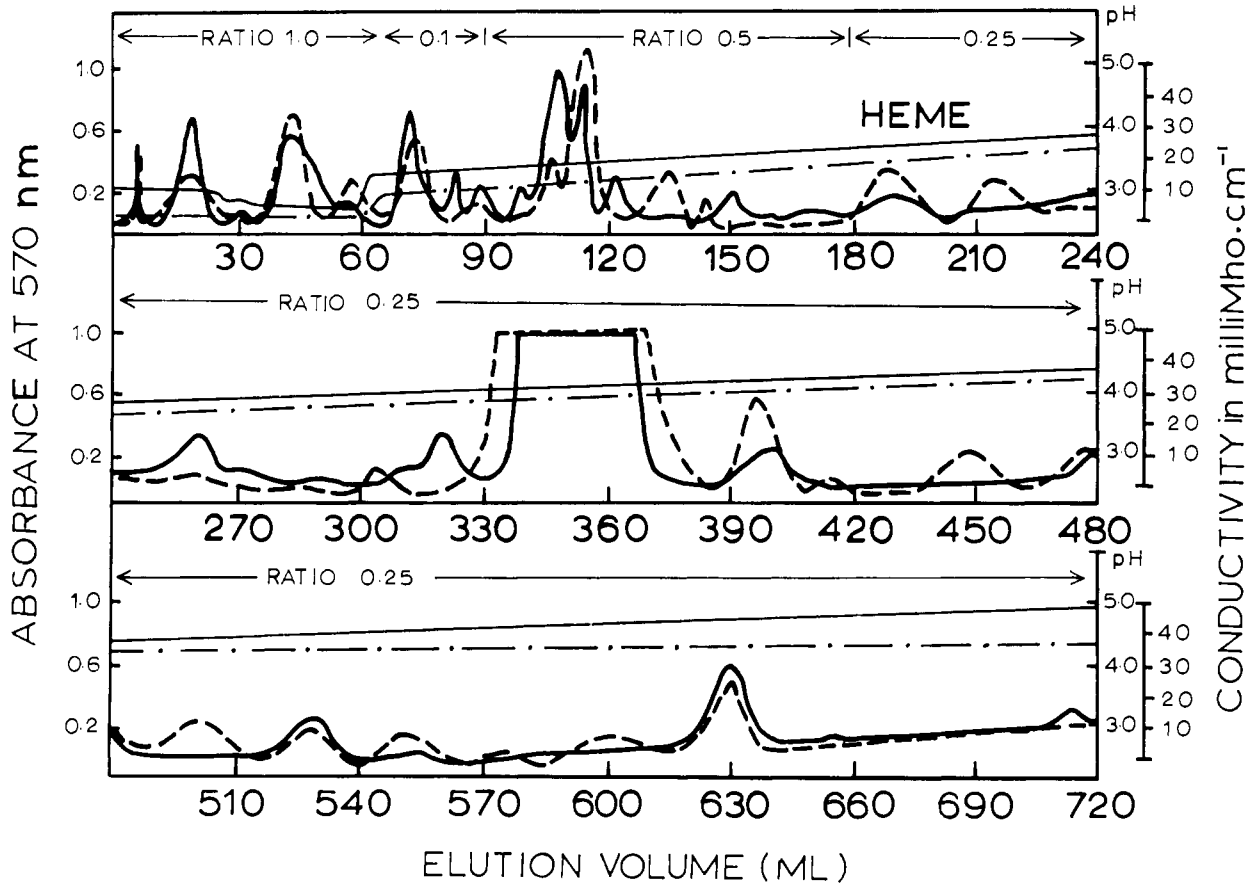

FIGURE 5: Comparisons of the elution patterns of the tryptic digests. The solid trace refers to the digest of the small *c*-type cytochrome while the dashed trace corresponds to the digest of cytochrome c_2. The digests (0.15 μmole) were applied to a column of cation-exchange resin (1.3 × 28.0 cm, PA-35). The peptides were then eluted with a linear gradient of pyridine-acetate made up from 400 ml of 0.2 M pyridine-acetate (pH 3.1) and 400 ml of 2.0 M pyridine-acetate (pH 5.0) at 55°, using a flow rate of 30 ml/hr. The column effluents were monitored at 570 nm, after reaction with ninhydrin, using a Gilford spectrophotometer, Model 2000, attached to the amino acid analyzer. A sensitivity ratio of 0.1 refers to a recorder setting of 1.0-A full scale.

(Figure 5). Despite this discrepancy, however, the digests of fractions I and II show a strong overall resemblance to each other in both peptide maps and column separation pattern of peptides. A large number of peptides appear in similar or nearly identical positions. The heme peptides were easily identified after these separations and appeared to chromatograph similarly in all cases.

In addition to these two *c*-type cytochromes, significant amounts of a cytochrome *c'* were also obtained by the initial extraction procedure. This heme protein was easily separated from the two smaller cytochromes by chromatography on either DEAE-cellulose or Sephadex G-75. Its isolation and characterization will be reported elsewhere.

Discussion

In contrast to the single type of cytochrome *c* found in all cell types of mammalian tissues it has become increasingly evident from studies of a variety of microorganisms (Perini *et al.*, 1964; Slonimski *et al.*, 1965; Bartsch, 1968; Kamen *et al.*, 1970) that lower forms of life have the ability to produce several soluble *c*-type cytochromes which cover a wide range of structural and functional properties. The two isocytochromes *c* of *Saccharomyces cerevisiae*, for instance, differ in primary structure, but exhibit substantial sequence homology (Stewart *et al.*, 1966, unpublished data as quoted by Sherman *et al.*, 1966) and are essentially indistinguishable with respect to physiological activities (Slonimski *et al.*, 1965). On the other hand, many *c*-type cytochromes found in bacteria are distinctly different in size, amino acid composition, light absorption spectra, oxidation–reduction potential, and biological function (Bartsch, 1968; Kamen *et al.*, 1970). This is particularly obvious in the case of the variant *c*-type cytochromes, called cytochromes *c'* or *cc'*, of photosynthetic bacteria (Dus *et al.*, 1962; Kamen *et al.*, 1963, 1970; Dus *et al.*, 1967a; Bartsch, 1968). But even the typical *c*-type cytochromes present in bacterial species, show great structural and functional diversity (Kamen *et al.*, 1970). The availability of reasonable quantities of two *c*-type cytochromes of *R. molischianum* from 4- to 5-day-old cells has therefore stimulated our work on a comparative structural study of these heme proteins.

From the amino acid analyses (Table I) it is well established that the same cell of *R. molischianum* synthesizes at least two distinct molecular forms of cytochrome *c* monomer, *i.e.*, a cytochrome c_2 and a small *c*-type cytochrome. The significance of the two minor subfractions of *c*-type cytochromes (Figure 1) is not clear at the present time, but they

may represent traces of fractions I and II which were deamidated or otherwise modified during the isolation and purification procedures.

The isolation of two *c*-type cytochromes from extracts of *R. molischianum* raises the question about the cellular origin of these heme proteins. Thus, the significance of the gradual increase in the relative concentration of cytochrome c_2 with time of culture growth is not understood at the present time. It is possible that this component represents a normally particle bound cytochrome which becomes increasingly more soluble with prolonged time of growth. At present, we do not know whether or not the structural genes for the two cytochromes are controlled independently. The investigation of this question is, however, complicated by the lack of techniques for isolation of well-defined subcellular particles of this organism and by the practical limitations for extended growth periods. Furthermore, the amount of heme proteins solubilized by our extraction procedure routinely does not exceed 50% of the total heme protein present. In this context, it should be mentioned that in *R. rubrum* (Kamen *et al.*, 1963) extended periods of growth lead to a substantial increase in the amounts of cytochrome c_2 that can be solubilized by our procedure. Thus, while the total amount of soluble *c*-type cytochromes of *R. molischianum* appears to increase with time of growth, it is not yet clear whether or not some of the increase in cytochrome c_2 occurs at the expense of the small *c*-type cytochrome.

The extinction coefficients of the reduced α bands of the native heme proteins and of the pyridine hemochromogens are both very similar for the two cytochromes and close to the values reported for bovine heart cytochrome *c* (Flatmark, 1964, 1967).

As shown in Table I, the two cytochromes are distinctly different in size and amino acid composition. Nevertheless, a certain degree of homology in their sequences has been observed. Dinitrophenylation revealed freely available amino termini, *i.e.*, alanine in both proteins. Sequential removal of five residues by Edman degradation indicated identical sequences for the amino-terminal portions of both proteins. A careful study of amino acid residues by carboxypeptidase A suggested that similarity may also exist between the C termini of these proteins. Furthermore, a comparison of the peptide patterns of tryptic digests of the two proteins (Figures 4 and 5) makes an overall similarity of the underlying sequences appear rather likely. Since column chromatography is more reliable than peptide maps, both in terms of resolution of components and with regard to quantitation of yields of peptides, we tend to believe that the excessive number of spots recognized on the paper probably includes many trace components.

The occurrence of homologous sequences in these two proteins is intriguing because similar relationships are likely to exist in many bacterial species containing more than one *c*-type cytochrome. Work to extend these structural comparisons to the entire amino acid sequences is now in progress.

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