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Purification and Properties of a *c*-Type Cytochrome from *Micrococcus denitrificans**

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ABSTRACT: A *c*-type cytochrome can be removed from intact cells of *Micrococcus denitrificans* by washing with weak buffer containing KCl, then with weak buffer. Under proper conditions the yield of the cytochrome can be 40% of the pigment obtainable from broken-cell extracts. Under these conditions only small amounts of contaminating protein and no other cytochromes are removed by the washings. Purification of the cytochrome *c* from the washings is a simple procedure. The cytochrome has an absorption spectrum similar to that of mammalian *c*-type cytochromes, but the ratio of dicarboxylic to diamino acids is twice as great in the bacterial pigment. Only one histidine is present. The molecular weight of *M. denitrificans* cytochrome *c* is slightly larger than that of the mammalian cytochrome (around 135 amino acids as compared to 104) and considerably larger than the *c*-type cytochrome which purified from *Pseudomonas aeruginosa*.

Although purified soluble cytochrome *c* and membrane-bound or purified cytochrome *c* oxidase of the respiratory chain system from different eukaryotic species will interact rapidly (plants, yeasts, molds, insects, vertebrates of all classes), the oxidases of most bacteria oxidize the eukaryotic cytochromes *c* very slowly, if at all. Conversely, most of the isolated bacterial cytochromes of the *c* type are not rapidly oxidized by mammalian oxidases (Smith, 1968). Some of the soluble *c*-type cytochromes from the bacterial respiratory chain systems have absorption spectra and redox potentials similar to those of the mammalian-type pigment, but the isoelectric points are often different (Smith, 1968). This is of interest in relation to the postulated electrostatic interaction involved in the reaction of oxidase preparations from mammalian sources with eukaryotic cytochromes *c* (Davies *et al.*, 1964; Smith and Minnaert, 1965). The complete amino acid sequence of only one respiratory chain bacterial *c*-type cytochrome is known, that from *Pseudomonas aeruginosa* (Ambler, 1963a,b). It resembles eukaryotic cytochromes *c* in several respects: the attachment of the heme group is the same; there are no free sulfhydryl groups in either; and the charge distribution between the heme and the amino terminus is rather similar in the two types.

A soluble *c*-type cytochrome was isolated from *Micrococcus denitrificans* by Kamen and Vernon (1955) and found to have an isoelectric point on the acid side of neutrality, in contrast to the mammalian pigment with an isoelectric point above pH 10 (Margoliash and Schejter, 1966; Flatmark and Vesterberg, 1966). In spite of this difference, the oxidase of *M. denitrificans* can oxidize both the bacterial and mammalian cytochromes at very rapid rates (Kamen and Vernon, 1955; Smith *et al.*, 1966) and the mammalian oxidase can oxidize the bacterial *c*-type cytochrome at a rather low rate (Kamen and Vernon, 1955; Smith *et al.*, 1966). Thus these activities of the *c*-type cytochrome and the oxidase differ from those of most other bacterial species. In order to make a further study of these reactions and gain more knowledge of the bacterial *c*-type cytochrome, a simpler method for isolating and purifying this pigment was devised. The method is described here, together with some of the properties and the amino acid composition of the cytochrome.

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Experimental Section

Isolation and Purification of c-Type Cytochrome. M. denitrificans ATCC 13543 were grown anaerobically in the presence of nitrate as described by Scholes and Smith (1968) for 12–20 hr beyond the phase of rapid growth. Large batches were grown either at the New England Enzyme Center or at Abbott Laboratories in North Chicago from an approximately 5% inoculum of cells grown anaerobically for 20 hr. Cells grown in large batches were collected by sedimentation in a Sharples centrifuge to the consistency of a wet paste. Some *c*-type cytochrome can be removed from the intact bacteria by washing with 0.01 M phosphate buffer, or with the same buffer containing KCl up to about 0.15 M. If washed with the latter, additional cytochrome can be removed by subsequent washing with 0.01 M buffer. As much as 40% of the cytochrome that can be obtained from broken-cell extracts can be removed from the intact cells by washing, together with only small amounts of contaminating protein. Purification of the cytochrome from the washings is a much easier procedure than that beginning with broken-cell extracts.

In practice the following procedure gave a good yield of cytochrome *c:* 500 l. of a culture grown for 40 hr in a fermenter at 28° yielded a wet paste equivalent to 300 g dry weight of cells. The paste was given three washes at 4° as follows: (1) 75 l. of 0.01 M phosphate buffer (sodium or potassium) (pH 7.0), (2) 20 l. of the same buffer containing 0.15 M KCl, and then (3) 50 l. of the 0.01 M phosphate buffer. The washing from step 2 was diluted 5-fold with cold water, then the three washings were pooled and adjusted to pH 6.0 by addition of acetic acid. DEAE-cellulose was added (1 g/l.) and the mixture stirred for 15 min at 4°. The DEAE-cellulose (Schleicher & Schuell) had been washed alternately with 0.5 M NaOH and 0.5 M HCl for at least five cycles, ending with the H^+ form, then twice with 0.1 M sodium acetate, adjusting the pH to 6.0 with that approximate salt concentration. Finally the DEAE-cellulose was washed several times with 0.01 M acetate buffer (pH 6.0). The cytochrome *c* is strongly adsorbed to the cellulose at low ionic strength at pH 6 and can be eluted with 0.25–0.3 M buffer at this pH. The DEAE-cellulose with adsorbed cytochrome *c* was collected by filtration and the cytochrome *c* eluted with 2 l. of acetate buffer per 100 g of cellulose. The eluted cytochrome was then dialyzed against five volumes of 0.02 M acetate buffer (pH 6.0) with four changes of buffer during 24 hr. The dialyzed cytochrome solution was concentrated by adsorbing on a short DEAE-cellulose column and eluting in a small volume with 0.5 M sodium acetate buffer. The bulk of the extraneous protein can be removed at this stage by making the solution saturated with ammonium sulfate. The manipulations up to this point should be completed as quickly as possible.

After overnight dialysis of the ammonium sulfate supernatant against several changes of 0.02 M acetate buffer of five volumes each, the cytochrome was adsorbed onto the top of a DEAE-cellulose column which was 2 × 36 cm and packed under 4 psi from nitrogen. The cytochrome was eluted with 1 l. of a linear gradient starting with 0.1 M acetate buffer (pH 6.0) and going to 0.5 M acetate buffer, pumped at the rate of 30 ml/hr into a fraction collector, collecting samples of 10 ml. The central part of the cytochrome peak was pooled, dialyzed, concentrated on a small, densely packed DEAE-cellulose column, and then eluted with strong buffer as before. Finally the cytochrome was rechromatographed on a Sephadex G-75 column (2 × 210 cm) run at room temperature with 0.05 M sodium borate (pH 8.0) pumped at the rate of 60 ml/hr. Rechromatographing the central peak from the Sephadex run on DEAE-cellulose resulted in a slight but significant increase in purity.

TABLE I: Absorbance of Fractions during Cytochrome *c* Purification.

Fraction	$A_{550\ nm}$ (Reduced) × Vol	$A_{550\ nm}$ (Reduced)/ $A_{280\ nm}$ (Oxidized)
Combined washings from bacteria	1017	0.113
Supernatant solution from $(NH_4)_2SO_4$ precipitation	224	1.11
Solution after DEAE and Sephadex chromatography		>1.2

Pre- and postpeak tubes from both chromatographies that show any cytochrome color can be reworked in the same manner to obtain maximal yields. Spectrophotometric measurement of the $A_{550\ nm}$ (reduced)/ $A_{280\ nm}$ (oxidized) (A_{550}/A_{280}) is an index of the purity of the preparation (Margoliash and Schejter, 1966); the $A_{550\ nm}$ is a measure of the cytochrome, while the $A_{280\ nm}$ indicates the total protein. After the final chromatography on DEAE-cellulose, the ratio was always greater than 1.2 in our hands. The efficiency of the chromatographic steps generally depends upon the amount of noncytochrome protein washed out of the bacteria with the cytochrome, and this, in turn, seems to vary with the age of the culture. The time recommended above for harvesting the culture gives a good yield of the soluble cytochrome *c* without much contaminating protein. Table I shows the change in the A_{550}/A_{280} ratio at several steps during the purification procedure.

Estimations of the Purity of the c-Type Cytochrome. ADDITIONAL SEPHADEX CHROMATOGRAPHY. When the cytochrome purified as described above was put through a column of Sephadex G-75 again (2.5 × 210 cm column with 0.05 M borate buffer, pH 8), and the protein peak collected in a fraction collector in 10-ml tubes, there was no increase in purity. The ratio $A_{550\ nm}/A_{280\ nm}$ remained constant at 1.2 across the nearly symmetrical peak. This ratio is similar to that of the mammalian pigment (Margoliash and Schejter, 1966).

ELECTROPHORESIS ON POLYACRYLAMIDE GEL. This procedure also showed no evidence of contamination and gave an estimated molecular weight of 14,100 by comparison to proteins of known molecular weight. The method used was that of Weber and Osburn (1969).

Properties of the c-Type Cytochrome. ABSORPTION SPECTRA. The absorption spectra of the oxidized and reduced forms (reduced with sodium borohydride) are given in Figure 1; they are almost identical with those of horse heart cytochrome *c*. The millimolar extinction coefficient of the reduced pigment at 550 nm is 26.8, calculated from the absorbance of samples of measured iron content.

IRON CONTENT. This was measured on a preparation which had been passed through a Sephadex G-75 column a second time and treated with an additional chromatography on DEAE-cellulose as described above to remove any possible

TABLE II: Approximate Amino Acid Composition of *M. denitrificans* Cytochrome *c*.[a]

	μmole of Amino Acid Recovered					
	24 hr	48 hr	72 hr	Best or Av	Best Value/ 0.073[b]	Nearest Integer
Lysine	(0.1146)	0.1191	0.1181	0.1186	16.21	16
Histidine	0.00689	(0.00802)	0.00687	0.00688	0.94	1
Arginine	(0.00692)	0.00839	0.00805	0.00822	1.12	1
Aspartic acid	0.1350	0.1332	0.1333	0.1338	18.32	18
Threonine	0.05620	(0.05230)	0.05531	0.05575	7.63	8
Serine	0.0207	0.0195	0.0183	0.0224	3.06	3
Glutamic acid	0.1198	0.1203	(0.1109)	0.1200	16.43	16
Proline	0.0564	0.0560	0.0573	0.0565	7.73	8
Glycine	0.1244	0.1251	(0.1211)	0.1247	17.08	17
Alanine	0.1119	0.1119	(0.1088)	0.1119	15.32	15
Valine	0.0384	0.0456	0.0487	0.0487	6.67	7
Methionine	0.0296	0.0303		0.0300	4.10	4
Isoleucine	0.0387	0.0426	0.0426	0.0426	5.83	6
Leucine	0.0463	0.0446	0.0448	0.0452	6.19	6
Tyrosine	0.0214	0.0224	0.0219	0.0220	3.01	3
Phenylalanine	0.0305	0.0310	0.0299	0.0304	4.16	4
						133
Cysteine						2
						135

[a] Serine values were extrapolated to zero time; values at 72 hr were used for valine and isoleucine. Values in parentheses were omitted from the calculations. Values for cysteine are not given, since recovery is incomplete by the method used. [b] 0.073 determined by method of Ambler (1963a,b).

contaminants which may have remained. The iron was assayed by the method of Cameron (1965) on samples dried to constant weight at 110°. Values obtained with three samples were 0.388, 0.398, and 0.386% iron (average 0.391). This would correspond to a molecular weight of 14,287, assuming one iron per molecule.

MOLECULAR WEIGHT ESTIMATED BY EQUILIBRIUM CENTRIFUGATION. The pure cytochrome *c* in 0.1 M sodium acetate was subjected to equilibrium centrifugation in the Beckman Model E ultracentrifuge, following the method of Yphantis (1960). The molecular weight was calculated assuming that the value of $\bar{v}$ is the same as that of horse heart cytochrome *c*, 0.725 (Margoliash and Lustgarten, 1962). The values obtained were 14,024, 14,478, 13,546, and 14,428 with four different dilutions of the cytochrome *c* solution, giving an average value of 14,119.

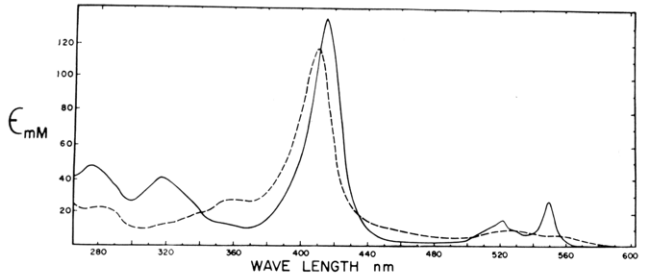

FIGURE 1: Absorption spectra of ferri- and ferrocytochrome *c*. The ferric form was measured in the presence of ferricyanide and the ferrous form in the presence of sodium borohydride, with equivalent concentrations of the oxidant or reductant in the blank cuvet.

AMINO ACID COMPOSITION. The amino acid composition of acid hydrolysates (6 N HCl, 110°, *in vacuo*) was estimated using a Beckman Model 120C amino acid analyzer, following the method of Spackman (1960). Table II lists the amino acid contents (except for tryptophan) found in samples hydrolyzed for 24, 48, and 72 hr. The heme was not removed from the cytochrome before hydrolysis. Calculation of a summation of the component amino acids, assuming no tryptophan, but two cysteine and one heme per molecule, results in a molecular weight of 14,861.

CRYSTAL FORM. After all of the work described above was completed, crystals of the cytochrome *c* were obtained

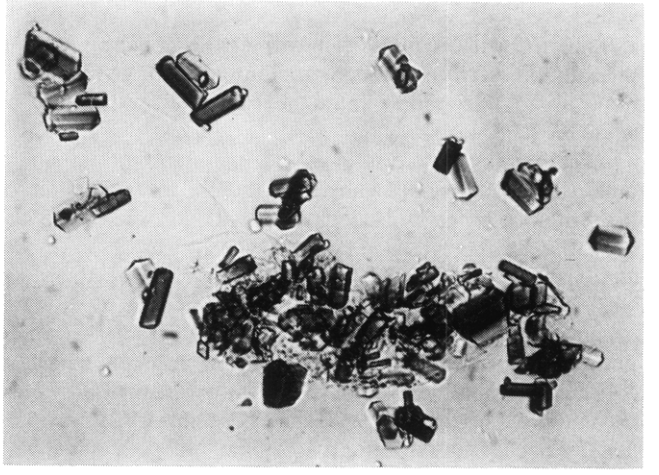

FIGURE 2: Crystals of ferricytochrome *c* (*M. denitrificans*) ×775.

from the purified preparation, using the same method followed by Margoliash and Walasek (1967) to crystallize mammalian cytochrome *c*. Figure 2 is a picture of the crystals.

Discussion

It is not clear why the *c*-type cytochrome can be removed from unbroken cells of *M. denitrificans* grown into the stationary phase by washing with dilute salt solutions or why one washing with stronger salt releases more cytochrome on subsequent washing with weak salt. About 40% of the cytochrome which is obtainable from broken-cell extracts can be removed by the washing procedures described. It is possible that mild osmotic shock is involved. But it also seems likely that the cytochrome is bound to the membranes of these bacteria primarily by electrostatic linkages, as is the cytochrome *c* of animal mitochondria (Jacobs and Sanadi, 1960). The other cytochromes of the bacteria are not removed during the washing procedure under the conditions described. For purification purposes it is fortunate that much of the *c*-type cytochrome can be isolated in this way, since we found it more difficult to purify the cytochrome from broken-cell extracts, as also did Vernon (1956). The washings that are the starting point of the preparation described here do not have large quantities of other proteins (the ratio A_{550}/A_{280} may increase only around 10-fold during purification (Table I), unless the bacteria are allowed to grow for longer periods than that recommended).

The procedure used to purify the cytochrome from the washings is simple, involving only adsorption onto and elution from DEAE-cellulose (batchwise and on columns), precipitation of contaminating proteins with ammonium sulfate and Sephadex chromatography. The extent of these procedures required depends upon the quantity of extraneous protein extracted from the bacteria, and this can be readily ascertained by measurements of the A_{550}/A_{280} ratio. After obtaining a preparation with an absorbance ratio of about 1.20, passage through Sephadex G-75 does not appear to increase the purity, and the cytochrome is eluted in a nearly symmetrical protein peak. Cytochrome preparations with such ratios were also pure by the criteria of electrophoresis on cellulose acetate strips or on polyacrylamide gel, and the cytochrome can be crystallized.

The absorption spectra of the ferric and ferrous forms of the *M. denitrificans* *c*-type cytochrome are similar to those of the mammalian pigments as to positions and ratios of the absorption peaks. However, the molar extinction coefficients are lower. The millimolar extinction coefficient of the ferrous form at 550 nm is 26.8, as compared to a value of 27.6 for horse ferrocytochrome *c* (Margoliash and Schejter, 1966).

The molecular weight of the *M. denitrificans* cytochrome *c* is considerably higher than that of the soluble cytochrome *c* isolated from *Pseudomonas fluorescens*, which is around 9000 (Ambler, 1963b). It is slightly larger than that of the mammalian pigment (mol wt 12,400).

The amino acid analysis shows the presence of a large number of dicarboxylic and diamino acids; the large preponderance of the former over the latter explains why the isoelectric point of this cytochrome is on the acid side of neutrality. The ratio of diamino to dicarboxylic amino acids is about the same in the *c*-type cytochromes from *M. denitrificans* and *Pseudomonas fluorescens* (Ambler, 1963a) and one-half the ratio in horse heart cytochrome *c*. The two bacterial cytochromes are also alike in having only one histidine per molecule, so that in both only one coordinate bond between the heme iron and the protein could be with an imidazole side chain. The *M. denitrificans* pigment has four methionines, twice as many as both horse and the *Pseudomonas* cytochrome. Both of the bacterial pigments have relatively high contents of tyrosine and phenylalanine and of glycine. It is difficult to make any more detailed comparisons of the two bacterial cytochromes because of the large difference in molecular weight. Significant comparisons of the *M. denitrificans* cytochrome with those from other species will require knowledge of the amino acid sequence. The *M. denitrificans* cytochrome *c* should be an interesting one to study because of the ability of the bacterial and mammalian oxidases and *c*-type cytochromes to interact.

The oxidase of *M. denitrificans* can oxidize mammalian cytochrome *c* at rates greater than the mammalian oxidase does, and the mammalian oxidase can oxidize the bacterial cytochrome at a low rate (Smith *et al.*, 1966), in spite of the great difference in isoelectric point between the mammalian and the bacterial proteins. Thus, the overall charge on the cytochrome *c* does not appear to be the important factor in the interaction of the oxidases with the cytochromes, but rather the charge of some specific areas which may interact to bring groups undergoing oxidation and reduction into proximity.

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Chemical Studies on the Cyanogen Bromide Peptides of Rat Skin Collagen. Amino Acid Sequence of α1-CB4*

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ABSTRACT: The amino acid sequence of α1-CB4, cyanogen bromide peptide 3 from the NH_2 terminus of the α1 chain of rat skin collagen, was determined. The peptide contains 47 amino acids arranged in the form $(GlyXY)_{15}GlyHse$ with about four-tenths of the X and Y positions occupied by proline and hydroxyproline. The extent of enzymatic hydroxylation of proline (to form hydroxyproline) was approximately 90% for three residues of the imino acid but was apparently complete in the case of three others. All the prolines acted upon by the hydroxylase were in position Y of the repeating triplet. Two peptide bonds involving lysine and arginine resisted proteolysis by trypsin; these basic amino acids were found to be adjacent to proline and hydroxyproline, respectively. The arginylhydroxyproline peptide bond was slightly susceptible to the enzyme, but no evidence was found to indicate that the lysylproline bond could be cleaved at all. α1-CB4 contains a high proportion of arginine and lysine, relative to that of collagen, an observation consistent with the heavy banding of regions of collagen near its NH_2 terminus when preparations of segment long-spacing aggregates are stained with phosphotungstic acid and viewed by electron microscopy.

Studies from several laboratories have employed CNBr cleavage of α chains of collagen to systematically investigate the chemistry of this important biological molecule (see Butler, 1970a). With this procedure rat skin collagen has been subdivided into 14 CNBr peptides exhibiting differences in composition, chromatographic properties, and presumably amino acid sequence (Butler *et al.*, 1967; Fietzek and Piez, 1969). Eight of the CNBr peptides derive from the two (identical) α1 chains of the molecule and the remaining six derive from the α2 chain. Previous studies on the CNBr peptides have provided information about the amino acid sequence of the NH_2-terminal end of the α1 chain (Kang *et al.*, 1967; Bornstein, 1967; Butler, 1970a,b), the location and identity of intramolecular cross-links (Bornstein and Piez, 1966; Bornstein *et al.*, 1966), the site of the initial cleavage of the collagen molecule by tadpole collagenase (Kang *et al.*, 1966), the location and distribution of the carbohydrate in α1 (Butler, 1970a), the location and probable nature of hydroxylamine-sensitive bonds (Butler, 1969; Bornstein, 1970), and the degree of species variability of collagen (Bornstein and Kang, 1970; Bornstein and Nesse, 1970). Additionally, studies relating to the antigenicity of collagen (Michaeli *et al.*, 1969; Timpl *et al.*, 1970; Bornstein and Nesse, 1970) and the occurrence of a more primitive type (type II) of collagen in cartilage (Miller and Matukas, 1970) have been facilitated by use of CNBr peptides.

In this communication we present data that indicate the amino acid sequence of α1-CB4, CNBr peptide 3 from the NH_2 terminus of the α1 chain of rat skin collagen. This peptide consists of 47 amino acids[1] with relatively high levels of the basic amino acids (arginine and lysine) and of the imino acids (proline and hydroxyproline).

Experimental Section

Preparation of α1-CB4. Lathyritic collagen was prepared (Bornstein and Piez, 1966) and then, without prior fractionation of α chains, cleaved with CNBr. Collagen samples were dissolved in 70% formic acid at a concentration of 10 mg/ml and incubated with a hundredfold excess (relative to methionine) of CNBr at room temperature for 4 hr. The samples were desalted by gel filtration on columns of Bio-Gel P2 (Bio-Rad Laboratories) and lyophilized.

The CNBr peptides from whole collagen were separated by modification of a method used previously (Butler, 1970a). Samples, ranging from 100 to 300 mg, were dissolved in 15 ml of 0.02 M sodium citrate (pH 3.6) and applied to 2.5 × 30 cm columns of CM-cellulose (Whatman CM-32) at 40°. Columns were then eluted with 50 ml of 0.02 M sodium citrate (pH 3.6) and finally with a linear gradient formed from 1100 ml each of 0.02 M sodium citrate (pH 3.6)–0.02 M NaCl and 0.02 M sodium citrate (pH 3.6)–0.14 M NaCl. The effluent was continuously monitored with a Beckman DB-G spectro-

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[1] The original report concerning the CNBr peptides of α1 indicated that α1-CB4 contained 46 amino acids (Butler *et al.*, 1967). Data reported in the present communication indicate that the correct composition includes 16 glycines instead of 15 reported in the earlier paper. The present data are consistent with those reported by Miller *et al.* (1969) and Kang *et al.* (1969) for α1-CB4 from chick collagens.