Cytochrome *c* in *Hydrogenomonas eutropha*¹

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A *c*-type cytochrome from *Hydrogenomonas eutropha* was purified 150-fold by butanol extraction, ammonium sulfate precipitation, and column chromatography. Three distinct *c*-type cytochromes were recovered which did not bind with either carbon monoxide or cyanide and hence did not appear to be denatured. Polyacrylamide gel electrophoresis indicated that one protein was acidic and the other two were basic. The acidic cytochrome *c* had a sedimentation coefficient of 3.46. Its amino acid composition was not markedly different from other bacterial cytochromes, but relative to mammalian cytochromes *c* it was low in lysine, threonine, and isoleucine and high in alanine and valine.

Hydrogen bacteria are facultative autotrophs which obtain their energy autotrophically from the oxidation of molecular hydrogen. Members of the genus *Hydrogenomonas* use oxygen as the primary acceptor for hydrogen.

Type *c* cytochromes from various organisms have been investigated in comparative biochemical studies of proteins in an effort to learn more about the evolution of living organisms (14). Cytochrome *c* has also been studied intensively to define the exact relationship between structure and function of proteins. The reconstitution of native cytochrome *c* from the heme and the apo-protein by Sano and Tanaka (11) opens the way to the total synthesis of the protein.

The cytochromes from hydrogen bacteria have not been purified previously, but as representatives from autotrophic bacteria they are of obvious interest in any study of the comparative biochemistry of the cytochromes. The existence of cytochrome *c* in *H. eutropha* was first reported by Wittenberger and Repaske (13) in their "soluble" hydrogenase preparation. Spectroscopic examination of the crude extract also suggested the presence of cytochrome *b*₁, which is mainly associated with the particles (10); cytochrome oxidase also was reported.

Work on the hydrogen bacteria has been hindered chiefly by the dearth of bacterial cells, because considerable hazard is involved in the large-scale growth of the bacteria in the optimum H₂:O₂:CO₂ ratio of 70:20:10 (8, 10). This paper describes the growth of *H. eutropha* on a large

scale [500-gal (1,893 liters) fermentor] under a nonexplosive gas mixture, the purification of the *c*-type cytochromes, and the amino acid composition and some of the physical properties of the most highly purified cytochrome *c*.

MATERIALS AND METHODS

Culture and recovery of bacteria. A liquid culture of *H. eutropha* was obtained from R. Repaske. The culture medium which was essentially that used by Wittenberger and Repaske (13) contained the following macronutrients (per liter): 0.25 M potassium phosphate buffer (pH 6.3), 100 ml; NH₄Cl, 1.0 g; NaCl, 0.1 g; CaCl₂, 0.1 g; NaHCO₃, 1.0 g; MgSO₄, 0.05 g. Stock solutions of these salts at 100-fold concentration (50-fold in the case of NaHCO₃) were prepared and autoclaved before use. The following mixture of micronutrients also was added (per liter): Fe(NH₄)₂(SO₄)₂·6H₂O, 7.0 mg; H₃BO₃, 2.8 mg; ZnSO₄·7H₂O, 1.76 mg; CoCl₂·6H₂O, 0.8 mg; CuSO₄·5H₂O, 0.08 mg; MnCl₂·4H₂O, 0.08 mg; NaMoO₄·2H₂O, 1.0 mg. This micronutrient mixture was conveniently prepared by adding 50 times the given quantities of salts to 100 ml of water to which a drop of concentrated hydrochloric acid had been added. This solution was refrigerated and used without sterilization. Two ml of the micronutrient solution was added to each liter of culture medium.

The organisms were first grown at 30 C in 500-ml suction flasks fitted with a rubber stopper. Flasks containing 100 to 125 ml of fresh culture medium were inoculated with 5 to 10 ml of an old culture. The liquid culture, stored at 4 C for 3 to 4 months, could be revived readily to give normal growth after a few transfers in the 500-ml flasks. Contamination was not a serious problem with this organism. The flasks were evacuated and gassed with 70% hydrogen, 20% oxygen, and 10% carbon dioxide (commercial tank gases). The flasks were shaken vigorously on a rotary shaker, and fresh gas mixture was introduced

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about every 6 hr. The bacterial culture reached an optical density of 0.8 to 0.9 in 24 to 36 hr as measured at 660 nm in a square cuvette of 1-cm light path with a Hitachi Perkin-Elmer spectrophotometer.

A 400-ml culture was used to inoculate 7 liters of fresh medium in a 10-liter bottle. A gas mixture of 3% H₂, 5% CO₂, and 92% air, monitored by flow meters, was passed continuously into the medium through gas spargers at a rate of 0.5 liter of gas per liter of medium per minute. The effluent gas was piped out of the window. Growth with this nonexplosive gas mixture also required about 30 hr.

Two such cultures or a 15-liter culture served, in turn, as the inoculum for a 50-gal (189 liters) fermentor containing 120 liters of medium. Only the empty fermentor was autoclaved before addition of medium constituents. A continuous flow of the 3% H₂ gas mixture was maintained in the fermentor at 0.5 liter of gas per liter of medium per minute. After about 15 hr of growth at 30 C, the optical density of the culture reached 0.85, and the cells were harvested with a Sharples supercentrifuge. The yield of wet cell paste was 240 g.

To obtain a larger yield of cells, a culture was grown in a 500-gal (1,893 liters) fermentor. After the fermentor was sterilized empty, 1,700 liters of tap water (Madison city water) was introduced. About 0.5 hr before inoculation, the salts for the medium were added in the solid form, except for micronutrients which were previously dissolved. A culture from a 50-gal (189 liters) fermentor was added directly as the inoculum. The gas composition and flow rate were the same as were used in the smaller fermentation vessels. Six cylinders of hydrogen (1,200 ft³ total) and four cylinders of carbon dioxide (200 lb or 91 kg) were consumed. Incubation was continued for 17 hr at 30 C. The cells were harvested with a large DeLaval centrifuge used in conjunction with a Sharples supercentrifuge. The bacteria were obtained as a very thick paste; the yield was about 2 g of cell paste per liter of medium. The cell paste was stored at -20 C.

Assay of cytochrome *c*. Both the purity and quantity of cytochrome *c* were determined spectrophotometrically at room temperature in a Cary model 15 recording spectrophotometer with the reference cell containing buffer only. The ratio of absorbance at 552 or 553 nm (depending upon the position of the α band) of the reduced form to the absorbance at 280 nm (with no oxidizing or reducing agent added) was designated as purity index.

Protein assay. Protein was determined according to the method of Lowry et al. (4) with bovine serum albumin as the standard.

Isolation of cytochrome *c*. Cytochrome was extracted from the cells with *n*-butyl alcohol in a manner similar to that described by Tissières (12). Cells (500 g) suspended in 200 ml of water were blended with 300 ml of *n*-butyl alcohol; 300 ml of water was added and the suspension was blended again before sedimentation of the cells by centrifugation.

Pretreatment of ion exchangers. A weighed amount of diethylaminoethyl (DEAE)-cellulose (Whatman DE23) was stirred into 15 volumes of 0.5 N HCl. After standing for 30 min, the supernatant fluid was

decanted and the exchanger was washed with distilled water until the pH was 4. Then the DEAE-cellulose was stirred into 15 volumes of 0.5 N NaOH; it was allowed to stand for 30 min, and the supernatant fluid was decanted. The second treatment with 0.5 N NaOH was repeated and was followed by washing with distilled water until the supernatant fluid was nearly neutral. The DEAE-cellulose then was equilibrated with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0.

Carboxymethyl cellulose (CMC, Whatman CM32) was treated much like the DE23, except that the first treatment was with base, followed by acid, and the intermediate pH was about 8. The washed CM32 was equilibrated with 0.005 M phosphate buffer, pH 7.0.

Concentration of protein solutions. Effluents of DEAE and CMC columns were concentrated in a Diaflo model 50 ultrafiltration cell (Amicon Corp., Cambridge, Mass.) by use of membrane UM1 and 45 lb of H₂ pressure. The flow rate was about 50 ml/hr.

Complexes of cytochrome *c* with ligands. These experiments were performed in glass Thunberg cuvettes.

(i) **Complex with carbon monoxide.** Each cuvette was filled with 3 ml of suitably diluted cytochrome *c* solution. As a control, a sample of the cytochrome *c* solution was denatured by adjusting it to pH 13 with 2 N NaOH. A trace of Na₂S₂O₄ was added to each cuvette. The system was evacuated four times and filled with H₂. The spectrum of the reduced cytochrome *c* was read with a Cary 15 spectrophotometer. Then the cuvettes were evacuated and filled with CO. Each cuvette was gently shaken and the spectrum was taken after 10 min at room temperature. Although the carbon monoxide-ferro-cytochrome *c* compound has been reported to be light-sensitive, the lights in the laboratory and the intensity of the light beam in the spectrophotometer were not sufficient to produce any noticeable dissociation of the compound. The individual spectra measured as described were then compared.

(ii) **Complex with cyanide.** The contents of the main compartment of the cuvettes were identical with those in the first experiment (*above*). Into each sidearm, 0.3 ml of 0.1 M KCN was pipetted. After the addition of Na₂S₂O₄, the system was filled with H₂ and the spectrum of the reduced cytochrome *c* was taken. Then the cyanide solution was mixed with the cytochrome *c* solution and the spectrum was obtained after incubation for 1 hr at room temperature.

(iii) **Complex with azide.** Experiments were run in 1-ml quartz cuvettes open to the atmosphere. Control cuvettes included: 0.1 ml of 0.001 M K₃Fe(CN)₆ in 0.9 ml of 0.05 M phosphate buffer, pH 7.0 (solution I); 0.1 ml of 0.001 M K₃Fe(CN)₆ in 0.9 ml of 0.5 M NaN₃ in 0.05 M phosphate buffer, pH 7.0 (solution II); cytochrome *c* preparation in solution I. The experimental cuvette contained the cytochrome *c* preparation in solution II. The spectrum of the azide complex was read 1 hr after the solutions were mixed. The individual spectra of oxidized cytochrome *c* plus and minus NaN₃ and the spectra of the controls without cytochrome *c* were compared.

Sedimentation studies. A Spinco model E ultra-

centrifuge with ultraviolet optics was used to study the sedimentation velocity pattern of CMC fraction I. The total volume of the sample was 0.8 ml, and its OD_{260} was 1.2 in a 1-cm light path. The run was made at 59,780 rev/min at 18 C for 90 min. Photographs were taken automatically every 8 min. The film was read with a Cary model 15 spectrophotometer set up as a densitometer.

Polyacrylamide gel electrophoresis. The procedure and materials used were similar to those of L. Orstein and B. J. Davis (Manufacturers' information supplied by Distillation Products Industries, Rochester, N.Y., and Canal Industrial Corp., Bethesda, Md.) with the following modifications. Tris electrode buffer—Tris, 6.0 g; glycine, 28.8 g; water to 1 liter; adjusted to pH 8.3; diluted 10-fold with water before use. Imidazole electrode buffer—imidazole, 6.8 g; 14% acetic acid, 30 ml; water to 1 liter; adjusted to pH 6.7; diluted 10-fold with water before use. β -Alanine electrode buffer— β -alanine, 31.2 g; glacial acetic acid, 8.0 ml; water to 1 liter; adjusted to pH 4.5; diluted 5-fold with water before use.

Amino acid analysis. One ml of CMC fraction I (about 1.4 mg of cytochrome *c*) was hydrolyzed in a sealed evacuated tube with 1 ml of concentrated HCl for 22 hr at 115 C in an autoclave. The hydrolysate was dried in a rotary vacuum evaporator, redissolved in water, redried three times to remove HCl, and then was taken up with 1 ml of 0.2 M citrate buffer, pH 2.2. The amino acids were separated and analyzed on a Beckman-Spinco amino acid analyzer (6, 7).

RESULTS AND DISCUSSION

Culture of *H. eutropha*. A typical growth curve obtained from a culture of *H. eutropha* grown in a 500-gal fermentor is illustrated in Fig. 1. The generation time was 3.5 to 4.0 hr. In our largest culture, 3.4 kg of cell paste was obtained. In 10-liter bottle cultures the generation time often was 7 to 8 hr; the limiting factor was probably gas dispersion.

It was established that good growth could be obtained with 3% hydrogen (providing a non-explosive gas mixture) instead of the usual 70% hydrogen, and this fact made large-scale culture feasible. The rate of growth was highly dependent upon gas equilibration. Repaske (10) reported a cell yield of 8 g of wet paste per liter in a 15-liter culture in which effective aeration was achieved by pumping gas in a closed circuit.

Study of type C cytochromes from *H. eutropha*. A flow sheet of the method adopted for isolation and purification of the *c*-type cytochromes is given in Fig. 2. The purity and yield at each step are summarized in Table 1.

The selection of 70% saturation with ammonium sulfate to precipitate cytochrome *c* was based upon the observation that increasing the concentration of ammonium sulfate above 70% decreased the purity of the cytochrome recovered.

FIG. 1. Growth curve for *H. eutropha* in a 500-gal fermentor. Temperature, 30 C; 0.5 liter of gas (3% H_2 , 5% CO_2 , 92% air) per liter of medium per minute.

This precipitation did not increase the purity of the sample greatly, but it did reduce the volume.

The chromatographic separation of the 70% $(NH_4)_2SO_4$ precipitate on a DEAE-cellulose column is shown in Fig. 3. It is interesting that when the column was eluted with 0.01 M Tris buffer, pH 8.0, a brown band stayed on the top of the column. The brown band started descending when the eluting buffer was changed to 0.01 M Tris, pH 8.0, 1.0 M NaCl, as would be expected for a ferredoxin. The characteristic absorption of ferredoxin at 390 nm was not evident in the eluted fraction; if ferredoxin was present, it was masked by the strong sorbitol band of contaminating heme proteins. To date, ferredoxin has been found in bacteria only in anaerobic organisms, and it would be of special interest to detect its presence in an aerobe such as *H. eutropha*. The absorption spectra of fractions A, D, and H (A and D gave pink bands on the DEAE column, and H produced a brown band) were taken with a Cary model 15 spectrophotometer; the data of interest are summarized in Table 2.

The cytochrome *c* preparations up to this stage remained in the reduced form, because the addition of $Na_2S_2O_4$ did not intensify their absorption peaks.

The concentrated DEAE fraction A then was separated on a microgranular CMC column (Fig. 4). The various fractions were assayed spectrophotometrically (Table 3). The absorption spectrum of fraction I is shown in Fig. 5. The cytochrome *c* became partially oxidized after the CMC treatment. An attempt was made to run an anaerobic column, but the cytochrome *c* from this column also was partially oxidized.

FIG. 2. Flow sheet for isolation and purification of type-c cytochromes from *H. eutropha*.

The purification steps were performed in succession without unnecessary delays. Storage of the samples for over a week before the DEAE treatment irreversibly changed the solution from pink-red to orange-brown. CMC fraction I was slowly autoxidizable upon storage at 4 C. The rate of autoxidation was much lower for fractions IV and V.

The appearance of multiple pink bands with essentially identical absorption spectra on both DEAE and CMC columns was puzzling. Were these different species of *c*-type cytochromes or were they simply artifacts of the preparation? Margoliash and Schejter (5), in their review on mammalian cytochrome *c*, commented, "Nearly every investigator who has chromatographed

TABLE 1. Summary of purification of type-*c* cytochromes from *H. eutropha*

Main steps	Volume	Purity index ^a	Yield
	ml		%
Crude extract	1,050	0.006	100.0
(NH ₄) ₂ SO ₄ fraction	80	0.02	40.0
DEAE cellulose	7 ^b	0.38	25.0
CMC			
Fraction I	2.0 ^b	0.94	1.9
Fraction IV	1.7 ^b	0.16	4.0
Fraction V	2.5 ^b	0.58	5.3

^a Purity index equals ratio of absorption of reduced form at 552 nm to absorption with no oxidizing or reducing agent added at 280 nm.

^b Volumes were reduced by concentration in the Diaflo apparatus.

FIG. 3. Chromatographic separation of the 70% ammonium sulfate precipitate on DEAE cellulose. Column size, 5.0 × 28.5 cm. Sample, 48 ml (15.2 mg/ml) of 70% (NH₄)₂SO₄-precipitated material, equilibrated with 0.01 M Tris buffer, pH 8.0. Flow rate, 2.5 to 3.0 ml/min. Room temperature. Elution schedule: initial, 0.01 M Tris buffer (pH 8.0); after fraction A, 0.01 M Tris buffer (pH 8.0), 0.1 M NaCl; after fraction E, 0.01 M Tris buffer (pH 8.0), 1.0 M NaCl. The pattern of elution was followed with a strip chart recorder connected to a spectrophotometer which monitored protein concentration at 280 nm; optical density is plotted. Cytochrome *c* was found mainly in A and D. H was the brown, "ferredoxin-like" fraction.

preparations of cytochrome *c* on cation-exchange resins has observed the presence of more than one fraction. Two of these, the reduced and oxidized forms of the protein, are naturally occurring. All the other chromatographic forms, with the single exception so far of the two isocytochromes *c* observed in baker's yeasts, are artifacts of the preparation." The modified varieties of cytochrome *c* consist of aggregated forms, monomeric materials in which one or more of the several amide groups

in the protein have been lost, and aggregates of cytochrome *c* with other proteins.

In an effort to distinguish between the native and denatured cytochromes *c*, the various frac-

TABLE 2. Spectral data for three fractions from the DEAE cellulose column^a

Determination	Fraction					
	A (110 ml)		D (80 ml)		H ^b (320 ml)	
	nm	OD	nm	OD	nm	OD
α Band	552-3	0.24	552-3	0.058		
β Band	524	0.18	524	0.050		
Soret	416	1.45	416	0.35	402-16	0.16
δ Band	315	0.35	315		315	
Protein	280	0.78	280	0.30	280	2.00
Purity index		0.31		0.2		
α/β		1.3		1.16		
α/Soret		0.165		0.166		
Soret (+Na ₂ S ₂ O ₄)						
Soret (-Na ₂ S ₂ O ₄)		1.0		1.0		
Total OD ₅₅₂ ^c		26		4.5		

^a Visible spectra of the eluates in the absence and presence of Na₂S₂O₄ were essentially identical.

^b Data presented here are from the spectrum taken on fraction H concentrated 15-fold after elution from the column shown in Fig. 3.

^c Total OD₅₅₂ equals total volume of fraction × OD at 552 nm with 1-cm light path.

FIG. 4. Chromatographic separation of DEAE fraction A on CMC. Column size, 2.2 × 23 cm. Sample: 7 ml (16 mg/ml) of concentrated DEAE fraction A, equilibrated with 0.005 M phosphate buffer (pH 7.0). Flow rate: 0.5 ml to 0.75 ml/min. Room temperature. Elution schedule: initial, 0.005 M phosphate buffer (pH 7.0); after I, 0.05 M phosphate buffer (pH 7.0); at peak of VI, 1.0 M phosphate buffer (pH 7.0). Cytochrome *c* was found in fractions I, IV, and V (three distinct pink bands). The pattern of elution was followed with a strip-chart recorder connected to a spectrophotometer which monitored protein concentration at 280 nm; optical density is plotted.

TABLE 3. Spectral data for the various fractions from a carboxymethyl cellulose column

	Fraction							
	I		IV		V		VI	
	(2.2 ml) ^a		(1.7 ml) ^a		(2.5 ml) ^a		(50 ml)	
	nm	OD	nm	OD	nm	OD	nm	OD
Eluted form								
α, β	— ^b		— ^b		— ^b		— ^b	
Soret	414	0.9	415–6	1.1	415–6	1.26	414–6	0.4
Reduced form								
α	553–4	0.15	553–4	0.18	553–4	0.19	553–4	0.078
β	523–6	0.115	525	0.15	524	0.16	522–5	0.06
Soret	416	1.03	416	1.17	416	1.29	416	0.456
δ	315	— ^c	315	— ^c	315	— ^c	315	— ^c
Protein	280	0.16	280	1.14	280	0.32	280	0.25
Purity index		0.94		0.16		0.58		0.31
α/β		1.3		1.22		1.2		1.3
α/Soret		0.145		0.156		0.144		0.17
Soret (+Na ₂ S ₂ O ₄)		1.1		1.06		1.01		1.14
Soret (–Na ₂ S ₂ O ₄)								
Total 553 units		3.3		6.2		9.3		3.9
Cytochrome <i>c</i> (mg) ^d		1.3		2.7		3.2		1.7

^a Fractions I, IV, and V were concentrated from about 10, 15, and 15 ml, respectively. The spectra were taken on the concentrated material with 10-, 20-, and 20-fold dilution, respectively.

^b The α and β bands were distinct but were intensified by the addition of sodium dithionite.

^c A slight absorption peak at 315 nm.

^d The cytochrome *c* content was estimated from the α absorption by using the mammalian cytochrome *c* extinction coefficient 27.8 mm^{−1} cm^{−1} and assuming a molecular weight of 12,000.

FIG. 5. Absorption spectrum of cytochrome *c* (CMC fraction I) in 0.005 M phosphate buffer (pH 7.0). The spectrum was measured with a Cary model 15 recording spectrophotometer. (A) reduced with Na₂S₂O₄; (B) oxidized with K₃Fe(CN)₆.

tions in their reduced form were tested for their complexing ability with carbon monoxide and cyanide, as formation of these complexes is indicative of denaturation of the cytochromes. The

COFe⁺⁺ complex exhibited a diminished and diffused band around 530 nm and an intensified band at 414 nm, compared with the native soret band at 416 nm. This was similar to the CO complex of undenatured mammalian cytochrome *c*, whose soret band, however, did not undergo a shift to the lower wavelength (2). The CN[−]-Fe⁺⁺ complex showed a reduced α band absorption and a slightly enhanced β band absorption, both shifted about 1 nm to a higher wavelength, similar to the undenatured mammalian counterpart (2). It differed from the latter in that its soret band showed a slight decrease in intensity, instead of an increase.

DEAE fraction D and CMC fractions I, IV, V, and VI were reacted with CO and CN[−]. D displayed positive CO-CN[−] complex spectra, although not more than 50% of the complex was present. The spectra of the other fractions indicated a very low degree of complex formation with either reagent. Therefore, the CMC fractions were mainly in the native form by the criterion of complex formation with either CO or

CN⁻. The reactivities of these fractions with the cytochrome *c* oxidase from *H. eutropha* would provide a further test for the biologically active species.

An azide complex with the oxidized cytochrome *c* was also formed. The azide complex differed from the oxidized cytochrome *c* only in having a slightly stronger absorption at 411 nm. The solet band of the oxidized cytochrome *c* was at 410 nm. Butt and Keilin (2) described the mammalian counterpart. The absorption spectrum of the N₃⁻-ferri-cytochrome *c* is of the same pattern as that of the free ferri-cytochrome *c*. It only differs from the latter by the shift of the α , β , and γ bands about 10, 7, and 2 nm, respectively, towards the red end of the spectrum and by a slightly greater extinction coefficient of the solet band. Again, the data indicate that the recovered type-*c* cytochromes from *H. eutropha* were not denatured during separation.

A sedimentation velocity study was performed on CMC fraction I. The sedimentation pattern was photographed periodically, and the plotted results gave a sedimentation coefficient of 3.46. Estimation of the molecular weight was not feasible without diffusion data. The tracing of the boundary indicated that the sample was free from larger molecules but had about 20% contamination by smaller molecules. These smaller molecular substances were not removed by the Diaflo apparatus, because the Diaflo effluent showed no absorption at 280 nm. Appropriate use of gel filtration may effect further purification.

Polyacrylamide gel electrophoresis was run on samples obtained at the chief steps in the purification procedure. Samples were run at pH 8.3 and 6.7, both from anode to cathode and from cathode to anode. Cytochrome *c* bands were visible in the tubes before staining. The crude extract had more than 10 protein bands, and the ammonium sulfate fractionation did not reduce the number. In the DEAE sample, the number of bands decreased considerably. CMC fraction I produced one major band plus two faint bands corresponding to those of fractions IV and V. Similar cross-contamination was found in the latter two samples. In addition to pH 8.3 and 6.7, CMC fraction I also was run at pH 4.5 from anode to cathode for 30 min, and the pink band descended. From the results, the isoelectric points could be approximated: CMC fraction I, between pH 4.5 and 6.7; CMC fraction IV, around 8.3; and CMC fraction V, above 8.3.

The amino acid composition of CMC fraction I obtained by acid hydrolysis is presented in Table 4. For purposes of comparison, the amino acid compositions of man, yeast, and some bacterial cytochromes are included. The total number

TABLE 4. Amino acid composition of mammalian and bacterial type *c* cytochromes

Amino acid	<i>Hydrogenomonas eutropha</i>	<i>Pseudomonas aeruginosa</i> ^a	<i>Pseudomonas fluorescens</i> ^a	<i>Desulfohalobium</i> cytochrome <i>c</i> ^b	Man ^c	<i>Saccharomyces</i> ^c
Tryptophan			2		1	1
Histidine	2 ^d (2.0)	1	1	9	3	4
Lysine	6 (6.2)	3	8	22	18	16
NH ₃	10 (10.1)			11		
Arginine	3 (2.6)	1	1	1	2	3
Aspartic acid	7 (7.3)	7	8	13	3	4
Asparagine					5	7
Threonine	3 (3.1)	4	2	5	7	8
Serine	3 (3.2)	4	3	5	2	4
Glutamic acid	7 (7.4)	7	10	6	8	7
Glutamine					2	2
Proline	5 (4.8)	5	6	4	4	4
Glycine	9 (8.7)	9	7	10	13	12
Alanine	12 (11.9)	7	13	11	6	7
Half Cystine		1	2	8	2	3
Valine	6 (5.6)	4	7	9	3	3
Methionine	2 (2.0)	1	2	3	3	2
Isoleucine	4 (3.6)	3	3	0	8	4
Tyrosine	3 (3.0)	2	1	3	5	5
Leucine	5 (4.9)	4	4	3	6	8
Phenylalanine	3 (2.6)	2	2	2	3	4
Total ^e	80	65	82	114	104	108

^a Ambler (1).

^b Coval et al. (3). Two haems per molecule.

^c Margoliash and Schejter (5).

^d Amino acid analysis indicated that these two His might be 1-CH₃ His.

^e The total numbers of residues were computed without including NH₃.

of residues, exclusive of tryptophan (usually one is present in cytochrome *c*) and cysteine or cystine (usually two half cystines are present in cytochrome *c*), was 80, making the minimum molecular weight of the cytochrome *c* about 8,500. Compared with the mammalian and yeast counterparts, the *H. eutropha* cytochrome *c* was particularly low in lysine content, which was in agreement with its acidic isoelectric point. It was also low in threonine and high in alanine and valine relative to mammalian and yeast cytochrome *c*. Its isoleucine content was identical to that of yeast but half that of the cytochrome *c* of man. In marked contrast to the remarkable similarities in the amino acid composition of all the mammalian cytochromes *c*, there are considerable variations in bacterial cytochromes even in the same genus; for example, the cytochrome *c* from the two strains of *Pseudomonas* exhibited quite different amino acid compositions.

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