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CYTOCHROME *c*-556, A DI-HEME PROTEIN FROM *PSEUDOMONAS AERUGINOSA*

J. SINGH and D. C. WHARTON

Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

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SUMMARY

A procedure is described for the purification of cytochrome *c*-556 from *Pseudomonas aeruginosa*. The isolated hemoprotein exists as a dimer with a molecular weight of approximately 77200. The dimer can be dissociated into a monomeric species (or single polypeptide chain) of 40500 molecular weight by means of sodium dodecyl sulfate or 4 M urea. The amino acid composition demonstrates the presence of four half-cystine residues per 43000 molecular weight. Heme and iron analyses indicate that two *c*-type hemes are covalently linked to each polypeptide chain. The absorption spectrum of ferrocytochrome *c*-556 has a double α-band with a peak at 556 nm and a shoulder at 552 nm; the β-band appears at 521 nm and the Soret band at 420 nm.

The electron paramagnetic resonance spectrum of ferricytochrome *c*-556 contains the elements of two ferric iron species, one a low spin and the other a high spin form.

The function of cytochrome *c*-556 is obscure. The purified cytochrome does not react with *Pseudomonas* cytochrome oxidase nor with the *Pseudomonas* cytochrome *c*-551 or copper protein.

The properties of cytochrome *c*-556 indicate that it is probably not the same species as the cytochrome *c*-554 previously isolated from the same organism.

INTRODUCTION

In the course of purifying electron transfer components from *Pseudomonas aeruginosa*[1] a *c*-type cytochrome was observed with spectral properties suggestive of cytochrome *c*-558 from *P. stutzeri*[2] and cytochrome *c*-557 from *Alcaligenes faecalis*[3]. Although its function is obscure at present, it is apparent that this cytochrome belongs to a group of "split-α" cytochromes found in denitrifying bacteria. In this communication the purification and some properties of cytochrome *c*-556 are described.

EXPERIMENTAL

Materials

$(NH_4)_2SO_4$, Tris, and urea were the "ultra-pure" grade obtained from Schwarz-Mann, Orangeburg, N.Y. Sephadex G-100 and carboxymethyl Sephadex C-50 were

Abbreviation: PCMB, *p*-chloromercuribenzoate.

products of Pharmacia. Acrylamide (electrophoresis grade), bisacrylamide (*N,N'*-methylenebisacrylamide), *N,N,N',N'*-tetramethylenediamine, mercaptoethanol, and Bromophenol blue were purchased from Eastman. $Na_2S_2O_4$ was a product of Hardman and Holden, Miles Platting, Manchester, England. Sodium dodecyl sulfate, *p*-chloromercuribenzoate (PCMB), and horse heart cytochrome *c* (Type III) were obtained from Sigma Chemical Co. Benzidine·2HCl was purchased from Fisher Scientific. Bathophenanthroline (2,9-diphenyl-1,10-phenanthroline) and bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) were products of G. Frederick Smith Chemical Co. Crystalline bovine serum albumin (10 mg/ml sterile solution) was obtained from Armour Pharmaceutical Co., Chicago, Ill. Ovalbumin, pepsin, and trypsin were purchased from Nutritional Biochemical Corp. A mixture of standard amino acids was obtained from Beckman Instruments.

All other inorganic reagents were of analytical reagent grade.

Methods

Estimation of protein

Protein was determined by the method of Lowry *et al.*[4] or by the biuret method of Gornall *et al.*[5]. For the biuret method the hemoprotein was first oxidized with 0.01 ml of 30% H_2O_2, and the biuret reagent was added only after the H_2O_2 had been removed by vigorous shaking of the sample for 5 min on a Vortex mixer. Crystalline bovine serum albumin was employed as a standard for both methods.

Estimation of iron and heme

Total iron and non-heme iron were determined according to the extraction method of Doeg and Ziegler[6]. This method, in the case of total iron, gave results that compared favorably with those obtained using the wet-digestion method of Van de Bogart and Beinert[7].

Heme content of the pure cytochrome was determined from the absorbance of the reduced *minus* oxidized hemochrome that had been formed by the reaction with alkaline pyridine according to the method of Appleby[8].

Estimation of copper

Copper was determined with bathocuproine according to the procedure of Wharton and Rader[9].

Absorption spectra

Absorption spectra were recorded either with a Beckman Model DK-2A recording spectrophotometer or a Cary Model 14 recording spectrophotometer. Spectra recorded at liquid-N_2 temperature were obtained with a Cary Model 14 spectrophotometer equipped with a sample holder for immersion in liquid N_2 and with a scattered transmittance accessory.

Electron paramagnetic resonance spectra

EPR spectra were recorded with a Varian Model V4500 spectrometer with modifications for work at low temperatures described by Beinert and coworkers[10–12].

The spectrum shown in this paper represents a first derivative of the absorption curve. The *g* values here do not necessarily represent true *g* values but may be given as a means of quickly locating prominent points in the spectrum.

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis[13]. A Buchler analytical apparatus with a Heathkit Model IP-17

regulated power supply was used. The gels were stained for protein and destained by the method of Chrambach *et al.*[14]. Heme was detected in the gels as described by Gudat *et al.*[1].

Molecular weight by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was measured by the method of Weber and Osborn[15]. Crystalline bovine serum albumin, pepsin, trypsin, and horse heart cytochrome *c* were used as standards.

Preparative polyacrylamide gel electrophoresis was performed with a water-jacketed Pharmacia column, Type K25/45 (2.5 cm × 45 cm). Gels and electrode buffers were similar to those employed in the analytical system. The gel was formed by pouring 50 ml of a 7% gel into the column which was stoppered at the lower end and had been cooled to 3 °C by circulating a solution of 50% ethylene glycol from a refrigerated bath. After the gel had solidified 5 ml of a spacer gel were added to the top of the running gel. After the spacer gel had solidified the rubber plug was removed from the bottom of the column and the column was lowered into a reservoir containing the lower electrode buffer. After the space above the spacer gel had been filled with the upper electrode buffer the sample, in 1–2 ml of 10% sucrose, was delivered onto the top of the gel by means of a syringe attached to a polyethylene tube. Electrophoresis was carried out at 350 V and 7 mA until the red band of the cytochrome had migrated about one-fourth of the way through the running gel (usually about 24 h). Coolant at 3 °C was continually circulated around the column during the run. At the conclusion of the run, the gel plug was removed from the column and the cytochrome band was separated by slicing. The cytochrome *c*-556 was eluted by homogenizing the gel in 15 ml of distilled water at 4 °C followed by centrifuging the homogenate at 28000 rev./min in a Spinco No. 30 rotor for 15 min. If the pellet still contained red color, the elution procedure was repeated.

Sedimentation equilibrium

The molecular weight of the isolated cytochrome *c*-556 was determined by sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge according to the method of Yphantis[16]. Samples were centrifuged at a speed of 18000 rev./min and at 5 °C in an Yphantis cell placed in a Type AN-D rotor. Data were recorded by means of a photoelectric scanner at a wavelength of 280 nm.

Amino acid analysis

Amino acid composition was determined using a Beckman Model 120C amino acid analyzer. Hydrolysates were prepared in 2-ml ampules by adding to 2-mg samples of the cytochrome an equal volume of 12 M HCl, sealing the ampules *in vacuo*, and heating at 110 °C for 24, 48, and 72 h. The hydrolysates were analyzed on the amino acid analyzer according to the procedure of Spackman *et al.*[17]. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, according to the method of Hirs[18]. Tryptophan was determined by hydrolysis in 3 M *p*-toluenesulfonic acid in the presence of 0.2% 3-(2-aminoethyl) indole according to the procedure of Liu and Chang[19].

Purification procedure

Culture conditions for *P. aeruginosa* as well as the procedure for obtaining the crude cell extract and partially purified cytochrome *c*-556 were those described by Gudat *et al.*[1]. The fractions collected from the column of CM-Sephadex C-50, and containing cytochrome *c*-556 on the basis of their absorption spectrum, were

TABLE I

PURIFICATION OF CYTOCHROME *c*-556 FROM *PSEUDOMONAS AERUGINOSA*

Fraction	$A_{556\ nm}/ml$	*Vol. (ml)*	*Total units**	*Percentage recovery***	*Protein (mg/ml)*	*Total protein (mg)*	*Purification index*
1. Effluent of CM-Sephadex C-50	7.7	2.6	20.0	100	34.3	89.5	1.0**
2. Second eluate of preparative gel electrophoresis	1.3	10.0	13.0	65	2.7	27.0	2.16
3. $(NH_4)_2SO_4$, 50–65% saturation fraction	3.9	3.1	12.1	60.5	4.75	14.7	3.68

* Total units is expressed as A/ml multiplied by volume in ml and is used here to denote the total amount of cytochrome.

** Since it was not possible to estimate the content of cytochrome *c*-556 before elution from CM-Sephadex C-50 this step is taken as 100% and a purification index of 1.0.

concentrated about 15-fold by vacuum dialysis at 4 °C using a Schleicher and Schuell No. 100 collodion bag. Sucrose was added to the concentrated sample to a final concentration of 10% and the sample was run on the preparative column of polyacrylamide gel described earlier. The cytochrome *c*-556 eluted from the gel was dialyzed overnight against 0.01 M sodium phosphate, pH 6.0, concentrated 5-fold by vacuum dialysis, and subjected again to preparative polyacrylamide gel electrophoresis. The product of the second electrophoresis was fractionated with saturated $(NH_4)_2SO_4$ (4 °C and pH 7). The fraction obtained between 50 and 65% of saturation was dissolved in 0.01 M sodium phosphate, pH 6.0, and was dialyzed overnight against the same buffer to remove residual $(NH_4)_2SO_4$. Table I shows the overall yield and purification of cytochrome *c*-556. The second preparative electrophoresis and the final $(NH_4)_2SO_4$ fractionation removed some minor protein components which otherwise migrated very close to the cytochrome when subjected to analytical polyacrylamide gel electrophoresis.

Activity assays

Since it was observed that reduced cytochrome *c*-556 reacted with O_2 it was necessary to perform most assays for enzymic activity under anaerobic conditions. For these assays a dialysis bag tied at one end and fitted with a rubber serum cap at the other was immersed in 0.01 M sodium phosphate, pH 6.0. The dialysis medium was gassed with O_2-free N_2 for 8 h in a cold room at 4 °C. Purified cytochrome *c*-556 was made anaerobic by evacuation in a test tube fitted with a rubber serum cap. The cytochrome was reduced by injecting a few μl of a solution of $Na_2S_2O_4$. The reduced cytochrome was then transferred by means of a gas-tight syringe into the dialysis bag immersed in the O_2-free buffer and was dialyzed for 24 h with continuous gassing of the buffer. Following dialysis the reduced cytochrome was transferred anaerobically by means of a gas-tight syringe into an anaerobic cuvette containing the electron transfer components to be assayed. Reduction of these components was then monitored spectrophotometrically using an appropriate wavelength.

The reaction of cytochrome *c*-556 with mammalian NADH:cytochrome *c* reductase was also performed under anaerobic conditions. Assay conditions were similar to those described by Hatefi and Rieske[20].

RESULTS

Disc gel electrophoresis

Analytical polyacrylamide gel electrophoresis of purified cytochrome *c*-556 resulted in only a single band when stained for either protein or for heme as shown in Fig. 1B. When the cytochrome was electrophoresed according to the procedure of Weber and Osborn following incubation in sodium dodecyl sulfate–mercaptoethanol, one major band and a barely visible minor band were observed (see Fig. 1C). The major band, which also stained for heme, corresponded to a molecular weight of $40\,500 \pm 10\%$ when compared to the migration of standards that were run simultaneously (Fig. 2). The minor band, which was estimated to have an intensity that was less than 5% of the major band, corresponded to a molecular weight of about 70000. The protein was not dissociated further even when it was incubated in a 3-fold excess of sodium dodecyl sulfate–mercaptoethanol.

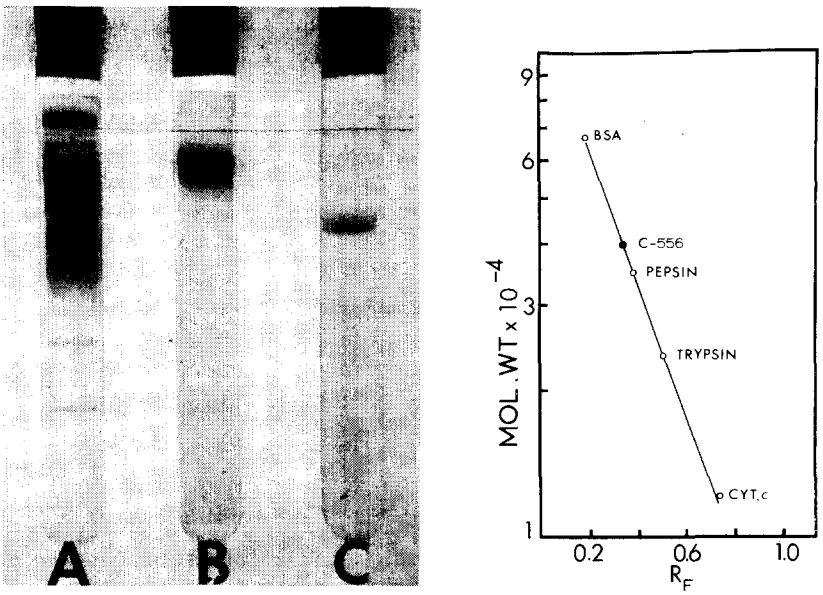


Fig. 1. Disc gel electrophoresis of cytochrome *c*-556. (A) Impure sample obtained after elution from a column of CM-Sephadex C-50. The most intense band is the cytochrome *c*-556. (B) Purified cytochrome *c*-556. (C) Purified cytochrome *c*-556 after incubation with sodium dodecyl sulfate–mercaptoethanol and electrophoresis according to the method of Weber and Osborn[15].

Fig. 2. Molecular weight analysis of cytochrome *c*-556 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. ○, protein standards (BSA= bovine serum albumin); ●, cytochrome *c*-556.

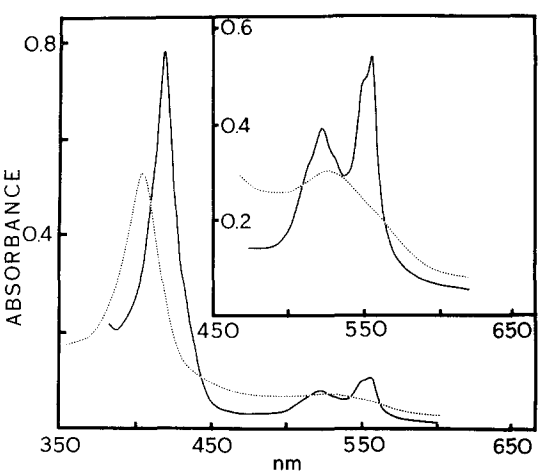
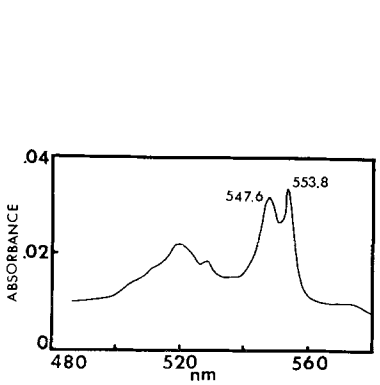


Fig. 3. Absorption spectra of purified cytochrome *c*-556. · · · · ·, oxidized; ———, reduced with a few crumbs of $Na_2S_2O_4$. The protein concentration was 0.7 mg per ml. For the complete spectrum, the sample was diluted 5-fold from that shown in the upper right corner.

Spectral properties

The absorption spectra of purified cytochrome *c*-556 taken at room temperature and at the temperature of liquid N_2 are shown in Figs 3 and 4, respectively. At room

temperature, reduced cytochrome *c*-556 shows an α-peak at 556 nm with a strong shoulder near 552 nm; the β-band is located at 521 nm and the Soret-band at 420 nm. The ratio of the absorbance of the Soret/α maxima is approximately seven, a value that is similar to most *c*-type cytochromes. As seen in Fig. 5, denaturation of the cytochrome with 6–8 M urea causes the peak at 556 nm to shift to 551.6 nm and results in an increase of about 12% in the absorbance at that wavelength. The original spectrum was recovered when the urea was removed by dialysis overnight. Unlike most *c*-type cytochromes that have a double α-band with a division of approximately 3 nm at the temperature of liquid N_2, the double α-band of cytochrome *c*-556 has a division of 6 nm.



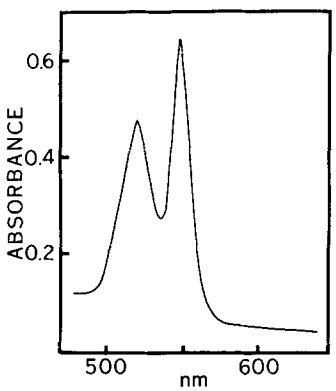


Fig. 4. Difference spectrum of purified cytochrome *c*-556 taken at the temperature of liquid N_2. The sample in water was taken to a concentration of 50% with respect to glycerol. Part of this sample was reduced with a few crumbs of $Na_2S_2O_4$. The reduced *minus* oxidized spectrum was recorded after devitrification.

Fig. 5. Absorption spectrum of purified reduced cytochrome *c*-556 in 6 M urea and at room temperature. The sample was reduced with a few crumbs of $Na_2S_2O_4$. The protein concentration was 0.7 mg per ml.

Identity of heme

When purified cytochrome *c*-556 was allowed to react with alkaline pyridine a *c*-type hemochrome was formed. On the basis of an ε of 22.3 $mM^{-1} \cdot cm^{-1}$ for the absorbance difference at 550 *minus* 553 nm, as given by Appleby[8], an equivalent weight of 21617 per mole of heme was calculated.

The heme could not be extracted from the cytochrome by acidified acetone but was cleaved from the protein using Ag_2SO_4 according to the method of Paul[21]. The cleaved heme was identified spectrophotometrically as hematoheme.

Iron analysis

No nonheme iron was detected in the purified cytochrome *c*-556 either by a direct assay or by subtraction of the heme content from the value for total iron. The heme content was found to be 0.258% by weight. On this basis the equivalent weight of the purified cytochrome was calculated to be 21650.

Amino acid composition

The amino acid composition of purified cytochrome *c*-556 is presented in

TABLE II

AMINO ACID COMPOSITION OF CYTOCHROME *c*-556

Amino acid	*Number of residues per half-cystine*			*Rounded* values*
	24 h	*48 h*	*72 h*	
Lysine	7.1	7.0	7.0	7
Histidine	3.0	3.0	2.9	3
Arginine	4.5	4.2	4.8	5
Aspartate + asparagine	9.9	9.3	9.8	10
Threonine	4.4	4.0	4.1	5**
Serine	5.8	4.9	4.8	6**
Glutamate + glutamine	11.1	10.6	11.3	11
Proline	6.7	6.5	6.9	7
Glycine	8.4	8.1	8.7	8
Alanine	10.4	10.0	10.6	10
Half-cystine	1.0	1.0	1.0	1***
Valine	6.6	6.5	7.0	7
Methionine	1.3	1.3	1.3	1***
Isoleucine	2.6	2.7	3.0	3
Leucine	8.1	7.9	8.4	8
Tyrosine	2.4	2.3	2.5	2
Phenylalanine	5.1	4.9	5.6	5
Tryptophan	0.3	—	—	0.3

* Rounded values represent the average of extrapolated values over 24, 48, and 72 h of hydrolysis rounded to the nearest integer.

** Values obtained by extrapolation to zero time from 24, 48, and 72 h of hydrolysis.

*** Half-cystine and methionine were estimated as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. The oxidized sample was hydrolyzed *in vacuo* at 110 °C for 20 h. The content of cysteic acid and methionine sulfone was extended to other hydrolyzed samples relative to aspartic acid.

Table II. The number of residues was calculated per half-cystine. An equivalent weight of 10780 was found per half-cystine, a value that agrees well with the finding that two heme groups occur per molecular weight of 43000, assuming that two half-cystine residues are required to bind one heme group covalently to the protein. Titration of the protein in 8 M urea with PCMB did not yield any reactive sulfhydryl groups.

Molecular weight

The molecular weight of purified cytochrome *c*-556 obtained by sedimentation equilibrium ultracentrifugation in 0.01 M sodium phosphate, pH 6.0, was 77200. A $\bar{v}$ of 0.729 was employed and was calculated from the amino acid composition. The data displayed in Fig. 6 demonstrate that only one macromolecular species was present. Centrifugation in the presence of 4 M urea and 0.01 M sodium phosphate, pH 6.0, showed only one macromolecular species but with a molecular weight of about 45000.

EPR spectroscopy

The EPR spectrum of cytochrome *c*-556 as seen in Fig. 7, contains the elements

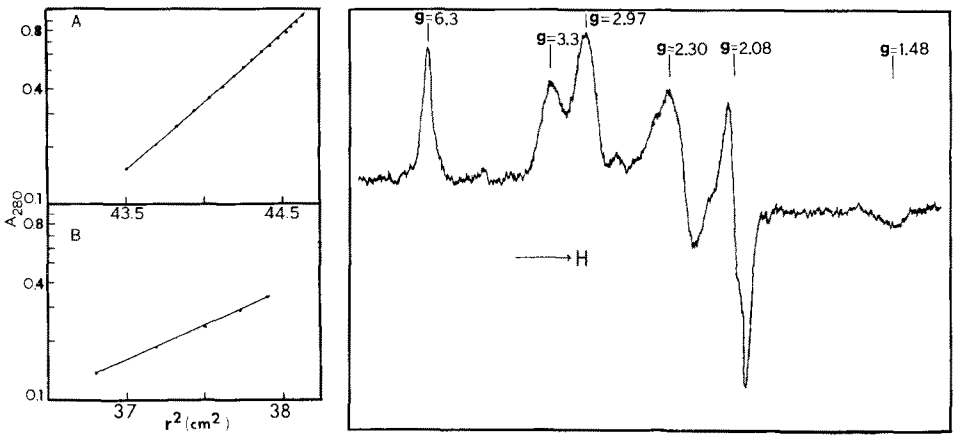


Fig. 6. Sedimentation equilibrium of purified cytochrome *c*-556 showing the relationship of the logarithm of the concentration of the cytochrome in 0.01 M sodium phosphate, pH 6.0, *versus* the square of the radius of the absorption maximum at 280 nm. (A) Untreated cytochrome; slope of 1.6 was calculated from these data. (B) The cytochrome in 0.01 M sodium phosphate, pH 6.0, and 4 M urea; slope of 0.86 was calculated from these data. The initial concentration of protein was equivalent to an absorbance of 0.25 cm^{-1} at 280 nm as determined in a Beckman Model DU-2 spectrophotometer.

Fig. 7. Electron paramagnetic resonance spectrum of cytochrome *c*-556. The spectrum was obtained using a Varian Model V-4500 spectrometer with modifications for work at the temperature of liquid He. The instrument settings were: power output, 0.3 mW; field modulation, 5 G; microwave frequency, approximately 9250 MHz; scanning rate, 250 G/min; temperature, 10 °K.

of several paramagnetic species. The element with a g value of 2.08 is probably due to the presence of contaminating copper, a hypothesis confirmed by chemical analysis. Two other components in the EPR spectrum probably result from heme iron. One of these is of the low spin ferric type with $g_z = 2.97$, $g_y = 2.30$, and $g_x = 1.48$. The other heme iron component appears to be of the high spin ferric type with g values at 6.3 and 3.3. These signals have been grouped together because there is some correspondence in their size with respect to the other group of signals from one preparation to another. Thus, small differences in the proportion of these two types occur from one preparation to another. The third g value in the second component has not been located. Furthermore, the association of the two sets of g values with a particular heme group of the cytochrome has not been determined. With the exception of the signal at $g = 2.08$, attributed to copper, the signals could only be observed near the temperature of liquid He.

Reactivity

Purified cytochrome *c*-556 was reduced by $Na_2S_2O_4$ or mercaptoethanol but not by ascorbate or dithiothreitol. The reduced cytochrome was oxidized by molecular O_2 but it did not appear to combine with CO.

The cytochrome did not react with *Pseudomonas* cytochrome oxidase either directly or *via Pseudomonas* cytochrome *c*-551 or the *Pseudomonas* copper protein. It was also unable to act as an electron acceptor for NADH:cytochrome *c* reductase prepared freshly from beef heart.

DISCUSSION

The cytochrome *c*-556 (552) purified to a state of macromolecular homogeneity from *P. aeruginosa*, resembles spectrally two other "split-α" *c*-type cytochromes previously isolated from denitrifying bacteria, namely cytochrome *c*-557 (551) from *A. faecalis*[2] and cytochrome *c*-558 (552) from *P. stutzeri*[3]. All three cytochromes exist in solutions as dimers with a molecular weight of about 75000. However, the cytochrome *c*-556 from *P. aeruginosa* differs from the other two cytochromes in that the monomeric species is a single polypeptide chain which contains two *c*-type heme groups linked covalently to the protein whereas the monomeric species of the other two cytochromes consists of only one heme group. This conclusion is supported by the observation that the equivalent weight on the basis of heme and iron content is 21 600 while the molecular weight of a single polypeptide chain is about 40 500 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Furthermore, this conclusion is strengthened by the finding that there is one half-cystine residue per molecular weight of 10 780.

During the course of the present studies it became necessary to compare cytochrome *c*-556 with another *c*-type cytochrome isolated from *P. aeruginosa* by Horio[22] and called cytochrome *c*-554. Although we have not been successful in identifying a cytochrome identical to cytochrome *c*-554 in extracts of cells of our culture of *P. aeruginosa*, a comparison of the properties of the two cytochromes suggests that they are dissimilar. In the first place, cytochrome *c*-556 is a "split-α" cytochrome whereas cytochrome *c*-554 is not. Secondly, cytochrome *c*-556 does not serve as an electron acceptor for mammalian NADH:cytochrome *c* reductase whereas cytochrome *c*-554 readily accepts electrons from that system.

It is not known with certainty whether the presence of two absorption maxima in the α-region of the spectrum of cytochrome *c*-556 is the result of the differing absorption properties of two different hemes or whether it results from the dimerization of the heme protein. Conditions which depolymerize the dimer, such as exposure to 6 M urea, also result in a reversible conversion of the α-region of the spectrum into a single symmetrical peak. However, the EPR spectrum suggests the presence of both high spin and low spin ferric iron species in the dimeric cytochrome. Whether these result from two particular heme groups in the protein or from the dimerization remains to be determined. In this regard it may be useful to examine the EPR spectra of the dimeric and "split-α" *c*-type cytochromes from *A. faecalis* and *P. stutzeri* since these contain only one heme group per monomer.

No function has yet been found for cytochrome *c*-556 nor, for that matter, for the somewhat similar cytochromes from *A. faecalis* and *P. stutzeri*. Although the purified cytochrome *c*-556 does not react directly with *Pseudomonas* cytochrome oxidase, cytochrome *c*-551, or the copper protein of *P. aeruginosa* it is possible that other components of the system may mediate electron transfer between it and the terminal acceptor system. Such mediators may remain attached to membrane fragments at the time that cytochrome *c*-556 is separated. The function of cytochrome *c*-556 may be elucidated when a better knowledge of the electron transfer chain of denitrifying bacteria is attained.

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REFERENCES

1 Gudat, J. C., Singh, J., and Wharton, D. C. (1973) *Biochim. Biophys. Acta*, 292, 376–390
2 Kodama, T. and Mori, T. (1969) *J. Biochem. Tokyo* 65, 621–628
3 Iwasaki, H. and Matsubara, T. (1971) *J. Biochem. Tokyo* 69, 847–857
4 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
5 Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* 177, 751–766
6 Doeg, K. A. and Ziegler, D. M. (1962) *Arch. Biochem. Biophys.* 97, 37–40
7 Van de Bogart, M. and Beinert, H. (1967) *Anal. Biochem.* 20, 325–334
8 Appleby, C. (1969) *Biochim. Biophys. Acta* 172, 88–105
9 Wharton, D. C. and Rader, M. (1970) *Anal. Biochem.* 33, 226–229
10 Beinert, H. and Kok, B. (1964) *Biochim. Biophys. Acta* 88, 278–288
11 Beinert, H. and Palmer, G. (1964) *J. Biol. Chem.* 237, 1221–1227
12 Palmer, G. (1965) in *Methods in Enzymology*, Vol. 10, pp. 594–609, Academic Press, New York
13 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
14 Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. (1967) *Anal. Biochem.* 20, 150–154
15 Weber, K. and Osborn, M. J. (1969) *J. Biol. Chem.* 244, 4406–4412
16 Yphantis, D. A. (1964) *Biochemistry* 3, 297–317
17 Spackman, D. H., Stein, W. H., and Moore, S. (1958) *Anal. Chem.* 30, 1190–1205
18 Hirs, C. H. W. (1967) in *Methods in Enzymology*, Vol. 11, pp. 59–62, Academic Press, New York
19 Liu, T.-Y. and Chang, Y. H. (1971) *J. Biol. Chem.* 246, 2842–2848
20 Hatefi, Y. and Rieske, J. S. (1965) in *Methods in Enzymology*, Vol. 10, pp. 225–231, Academic Press, New York
21 Paul, K. G. (1950) *Acta Chem. Scand.* 4, 239–244
22 Horio, T. (1958) *J. Biochem. Tokyo* 45, 267–279