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PURIFICATION AND PROPERTIES OF CYTOCHROME *c*-553, AN ELECTRON ACCEPTOR FOR FORMATE DEHYDROGENASE OF *DESULFOVIBRIO VULGARIS*, MIYAZAKI

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Summary

Cytochrome *c*-553 of *Desulfovibrio vulgaris*, Miyazaki, was purified to homogeneity. The absorption spectrum of the ferro form has four peaks at 553, 525, 417 and 317 nm with a plateau near 280 nm, and that of the ferri form has three peaks at 525, 410 and 360 nm with a plateau near 280 nm and a shoulder at 560 nm. The millimolar absorbance coefficient of the α-peak of the ferro form is 23.9. The molecular weight of cytochrome *c*-553 is 8000, and it contains one heme. Its isoelectric point is rather alkaline, and its standard redox potential is −0.26 V at pH 7.0. Its amino acid composition is unique; it lacks proline, isoleucine and tryptophan.

Ferrocytochrome *c*-553 does not combine with CO, nor does it transfer electrons directly to various redox carriers such as flavin nucleotides, methylene blue, indigodisulfonate, 5-methylphenazinium methyl sulfate, 1-methoxy-5-methylphenazinium methyl sulfate, viologens and cytochrome c_3, but is oxidized by ferricyanide or by O_2.

Cytochrome *c*-553 can be reduced by formate dehydrogenase of this bacterium in the presence of formate, but not by hydrogenase under H_2. The formate dehydrogenase does not reduce cytochrome c_3 in the presence of formate. The systematic name for formate dehydrogenase of *D. vulgaris* is, therefore, established as formate:ferricytochrome *c*-553 oxidoreductase in EC subclass 1.2.2.—.

Introduction

Cell-free extracts of *Desulfovibrio vulgaris* contain at least three kinds of *c*-cytochrome [1,2]. The first, cytochrome c_3, has an unusually negative redox

potential [1,3—6], contains four hemes [1,7], and is an electron acceptor for *Desulfovibrio* hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) [8,9]. The second is cytochrome *c*-553 [2,10—12]. This was characterized as an electron acceptor for formate dehydrogenase of this bacterium using rather unpurified preparations [10]. The third high molecular weight cytochrome of unknown biochemical function is present in the sonic extracts [1,2,7,12]. The particulate fraction of this bacterium also contains at least three kinds of cytochromes of unknown character [13]. This paper purports to describe the purification and properties of cytochrome *c*-553, and of formate dehydrogenase of this bacterium.

Materials and Methods

Desulfovibrio vulgaris, Miyazaki, was cultured as described before [9]. Bacterial sonicate was prepared by disintegrating wet cells of *D. vulgaris* suspended in 5—6 volumes of H_2O with an ultrasonic disintegrator (UR-200P, Tomy Seiko Co., Tokyo) at 20 kHz, 180 W, for 12 min. Cytochrome c_3 and hydrogenase were prepared from this sonicate as described before [1,14].

Erabutoxin *a* [15] was a generous gift from Prof. N. Tamiya of Tohoku University. Ribonuclease A (EC 3.1.4.22) and trypsin (EC 3.4.21.4) were products of Sigma. Carbon monoxide (99.8%) was from Takachiho Kagaku Kogyo Co., Tokyo. Tris-HCl buffer used in this study was of pH 7.3. It was made anaerobic by bubbling N_2 through a reservoir and going through a chromatographic column when used in the purification of formate dehydrogenase. QAE-Sephadex A-50 (Pharmacia) was washed several times with 0.1 M NaOH and H_2O and equilibrated with 0.01 M Tris-HCl. CM-cellulose (Serva) was washed with 0.2 M NaOH (twice), H_2O (several times), 0.2 M HCl (twice), H_2O (several times) and 0.2 M aqueous NH_4OH (twice) to prepare the NH_4^+ form, and was further washed several times with H_2O so that the pH of the final washing would be neutral. Amberlite CG-50 (NH_4^+-form) was prepared similarly.

Spectrophotometric measurements were made with a Hitachi 124 or Shimadzu UV-210A spectrophotometer equipped with a recorder. Purity index of cytochrome *c*-553 was defined as A_{553nm}(ferro form)/A_{280nm}(ferri form). In the early stages of the purification where A_{553nm} did not represent the net height of the α-peak, correction was made by subtracting background absorption near the α-peak. The concentration of cytochrome *c*-553 was measured spectrophotometrically by measuring the absorbance at the α-peak of its pyridine ferrohemochrome, whose millimolar absorbance coefficient was taken as 29.1 [16].

Disc electrophoresis was carried out as described in Ref. 17. Sodium dodecyl sulfate-gel electrophoresis was carried out as described by Weber and Osborn [18]. Amino acid analysis was carried out after hydrolyzing the protein with 6 M HCl in vacuo in sealed Pyrex glass tubes for 24 and 48 h. Corrections were made for destruction of serine and threonine by extrapolation to zero time of hydrolysis. Cysteine was determined as cysteic acid after performic acid oxidation [19] of the sample which had been deprived of heme moiety [20]. Tryptophan was determined colorimetrically [21], and iron content was

measured by atomic absorption spectrometry at 248.3 nm with a Jarrell Ash atomic absorption type AA-I MK-II spectrophotometer.

Formate dehydrogenase activity was assayed spectrophotometrically. A reaction mixture containing properly filuted enzyme and 15 μmol sodium formate in 2.9 ml 6.9 mM phosphate buffer, pH 6.5, was placed in a Thunberg type optical cell of 1-cm light path, and incubated anaerobically at 30°C. At zero time, 0.1 ml 5 mM 1-methoxy-5-methylphenazinium methyl sulfate solution [22] was added anaerobically to the cell, and the decrease in absorbance at 505 nm recorded. The change of one absorbance unit corresponds to the reduction of 1.056 μmol of the dye under these conditions. One unit of formate dehydrogenase is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol formate, i.e., the reduction of 1 μmol dye per min under these experimental conditions. Hydrogenase activity was assayed by the enzymic electric cell method [23]. Ascorbate oxidase activity was assayed as described by Dawson and Magee [24].

Results

Partial purification of formate dehydrogenase

Bacterial extract (48 ml) was prepared from 10.6 g wet cells of *D. vulgaris* by centrifuging the bacterial sonicate. Total activity and specific activity of this extract were 9.6 units and 0.0032 unit/A_{280nm}, respectively. Formate dehydrogenase was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation (31.3 g/100 ml). The precipitate was dissolved in 80 ml 0.01 M Tris-HCl containing 0.04 M NaCl, concentrated in a Diaflo cell with PM-30 membrane to 5 ml and chromatographed through a column (2.2 × 73.1 cm) of Sephadex G-200. The elution buffer was 0.05 M Tris-HCl containing 0.2 M NaCl. The active fractions eluted between 120 and 175 ml of the effluent were collected, concentrated and the buffer solution of the enzyme was substituted with 0.01 M Tris-HCl. The total and specific activities at this stage were 1.86 units and 0.036 unit/A_{280nm}, respectively. This was then applied to a column (1.2 × 10 cm) of QAE-Sephadex A-50, and the activity adsorbed on the column was eluted with 0.02 M Tris-HCl containing 0.08 M NaCl. The active fractions were collected and concentrated to 3.9 ml. The total and specific activities of this concentrate were 0.36 unit and 0.09 unit/A_{280nm}, respectively. Although it still contained some proteinaceous impurities revealable upon disc electrophoresis, the specific activity was 28 times as high as that of the original extract, and did not contain any hydrogenase activity. This was used as partially purified formate dehydrogenase.

Purification of cytochrome c-553

Bacterial sonicate (600 ml) prepared from 82 g wet cells of *D. vulgaris* was centrifuged at 80 000 × *g* for 60 min to separate the supernatant (524 ml) containing soluble cytochromes from precipitate. Total α-peak height and the purity index of this extract were 251 absorbance units and 0.0136, respectively. Since cytochrome *c*-553 occupied 5.4% of this total α-peak height, the net purity index of this extract was only 0.0007. This fluid was passed through an Amberlite CG-50 (NH_4^+) column (1.3 × 10 cm) to remove cytochrome c_3.

Total α-peak height and the purity index of the effluent was 126 and 0.0058, of which cytochrome *c*-553 occupied 11%. This was concentrated to 1/5 of its volume with a rotary evaporator at 40°C. Caution must be paid not to dry the content, since cytochrome *c*-553 has a tendency to coprecipitate with proteinaceous impurities, being formed during the concentration and removed by centrifugation. The concentrate was diluted with H_2O to 7.5 volumes and passed through a column (1.3 X 10 cm) of CM-cellulose (NH_4^+). All cytochrome components were adsorbed on this column and eluted with 0.02 M NH_4OH. The fractions containing cytochromes were combined, concentrated to 5.0 ml with a rotary evaporator at 40°C, and passed through a column (2.2 X 128 cm) of Sephadex G-50 (fine). The eluting buffer was 0.05 M Tris-HCl containing 0.2 M NaCl. The high molecular weight cytochrome and cytochrome *c*-553 were separately eluted as shown in Fig. 1. The yield of cytochrome *c*-553 at this stage was 87%. Fractions containing cytochrome *c*-553 were combined, dialyzed against H_2O for 20 h, and passed again through a column (1.3 X 10 cm) of CM-cellulose (NH_4^+). The column was washed with H_2O and the adsorbed cytochrome eluted with aqueous ammonia, the concentration of which was gradually raised from 0 to 0.02 M. Cytochrome *c*-553 was eluted between 0.005 and 0.01 M NH_4OH. The fractions containing cytochrome *c*-553 of the highest purity index, i.e., 1.3, were combined, concentrated in a rotary evaporator at 40°C, and the concentrate was dialyzed against H_2O (twice 5 l). The purity index of the final preparation was 1.3, and the overall yield 73% of the original extract. Disc electrophoresis of the final preparation gave a single band.

Spectral properties of cytochrome c-553

Absorption spectra of the ferri and ferro forms of cytochrome *c*-553 are illustrated in Fig. 2. The positions and millimolar absorbance coefficients (ϵ_{mM}) of the peaks are: 525—526 nm (10.6), 410 nm (109), 360 nm (27.8) and 277—280 nm (19.3) in the ferri form, and 553 nm (23.9), 524 nm (16.2), 417 nm (142) and 317 nm (32.1) in the ferro form. There are seven isosbestic points (560, 541, 535, 509, 437, 412 and 337 nm).

Heme content of cytochrome c-553

Concentration of a purified cytochrome *c*-553 preparation was first

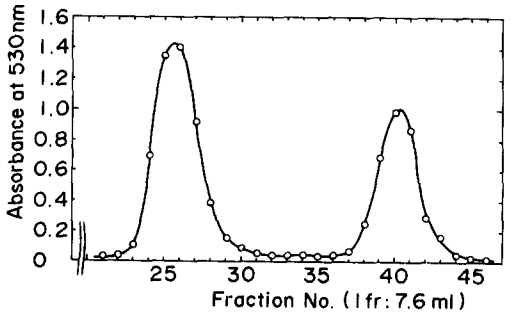


Fig. 1. Elution pattern of cytochromes from Sephadex G-50 (fine) column.

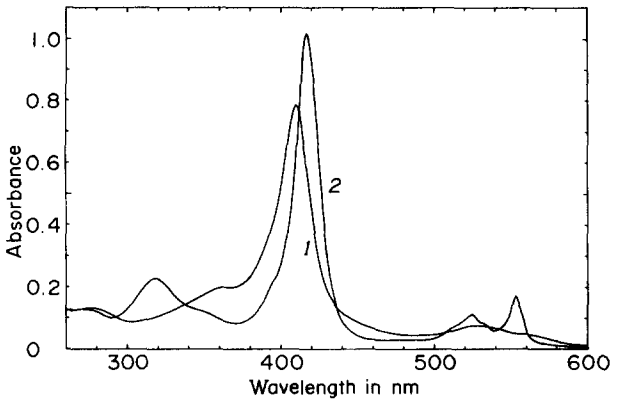


Fig. 2. Absorption spectra of the ferri and ferro forms of cytochrome *c*-553. A mixture containing 18.5 munits formate dehydrogenase, 5 mM sodium formate and 7.2 μM cytochrome *c*-553 in 3.0 ml 6.7 mM phosphate buffer, pH 6.5, was anaerobically incubated in a Thunberg type optical cell of 1-cm light path for 10 min, the spectrum recorded and corrected for the backgroun absorption caused by the formate dehydrogenase preparation. Cytochrome *c*-553 was not reduced in the absence of either formate or formate dehydrogenase. The reduction of the cytochrome was complete, since no further change was observed upon addition of $Na_2S_2O_4$. The spectrum of the ferri form was recorded ordinarily.

estimated by the pyridine ferrohemochrome method and found to be 175 μM. A 4.16-ml portion of this solution was frozen and lyophilized under reduced pressure at room temperature for more than 50 h. The lyophilized sample weighed 5.54 mg immediately after drying. Thus the heme content of this cytochrome was 1 mol per 7.6 kg dry weight giving a minimal molecular weight of 7600.

Another cytochrome *c*-553 solution (42.2 μM, estimated from its α-peak height of its ferro form) was analyzed for iron and found to contain 2.40 ppm, i.e., 43.0 μM.

Molecular weight of cytochrome c-553

This was estimated by SDS-gel electrophoresis. Erabutoxin *a* (M_w = 6850, Ref. 25), ribonuclease A (M_w = 13 700) and trypsin (M_w = 23 300) were run as molecular weight standards. Fig. 3 shows relative mobility of cytochrome *c*-553 and those of the standard proteins. From this the molecular weight of cytochrome *c*-553 was estimated to be about 8160, a value consistent with that obtained from the heme analysis.

Amino acid composition of cytochrome c-553

Amino acid composition of cytochrome *c*-553 of *D. vulgaris*, Miyazaki, is shown in Table I, for comparison together with that of cytochrome *c*-553 of *D. vulgaris*, Hildenborough, reported by Bruschi and Le Gall [12].

Isoelectric point of cytochrome c-553

Cytochrome *c*-553 and yeast cytochrome *c* were spotted on cellogel seats (Chemetron, Milano, Italy), immersed in buffer solutions of pH 9.6 and 10.2 and electrophoresed. Both migrated to the direction to the cathode. This suggests that the isoelectric point of cytochrome *c*-553 must be rather alkaline.

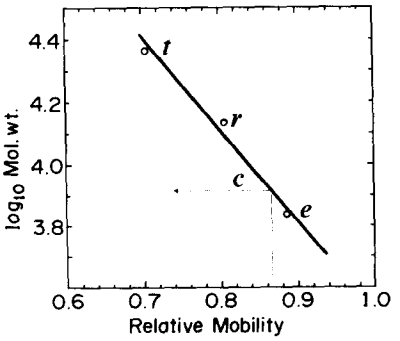


Fig. 3. Gel electrophoretic mobilities relative to that of bromophenol blue. *t*: trypsin; *r*: ribonuclease A; *e*: erabutoxin *a*; and *c*: cytochrome *c*-553.

Standard redox potential of cytochrome c-553

This was estimated from equilibration between a cysteine/cystine couple and cytochrome *c*-553 by the following equation:

$$E'_{c\text{-}553} - E'_{cys} = \frac{RT}{2F} \log_e \frac{[\text{cystine}][\text{ferrocytochrome}]^2}{[\text{cysteine}]^2[\text{ferricytochrome}]^2} \left(\frac{[H^+]}{10^{-7}}\right)^2$$

where R is the gas constant, T the absolute temperature, F the Faraday constant, and $E'_{c\text{-}553}$ and E'_{cys} the standard redox potentials of cytochrome *c*-553 and a cysteine/cystine couple at pH 7.0, respectively.

Cytochrome *c*-553 solution was added to a reaction mixture containing various amounts of cysteine and cystine in 0.02 M phosphate buffer and left

TABLE I

AMINO ACID COMPOSITION OF CYTOCHROME *c*-553 OF *D. VULGARIS*

	Miyazaki strain		Hildenborough strain number
	%	number	(Ref. 12)
Lys	15.83	11	12
His	2.51	2	1
Arg	1.27	1	1
Asp + Asn	8.15	6	5 + 1
Thr	1.69	1	1
Ser	5.61	4	6
Glu + Gln	7.68	5	5 + 1
Pro	0.00	0	1
Gly	13.80	9	12
Ala	14.29	10	14
Cys	2.86	2	2
Val	6.80	5	2
Met	6.00	4	6
Ileu	0.29	0	1
Leu	6.39	5	5
Tyr	5.81	4	6
Phe	0.99	1	0
Tryp	0	0	0
Total		70	82

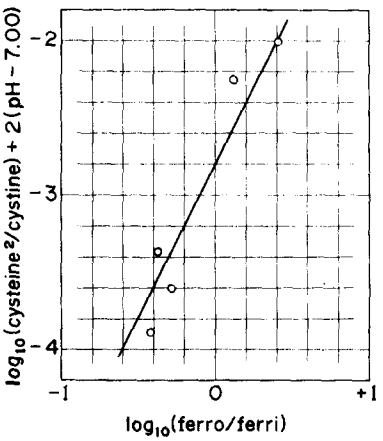
standing anaerobically in an optical cell to attain equilibrium. Then the percentage of the ferri and ferro forms of cytochrome *c*-553 were estimated spectrophotometrically and the pH of the solution was recorded. Fig. 4 shows the plot of the results. From the intersection of the line at the ordinate, the standard redox potential of cytochrome *c*-553 was calculated to be +0.084 V more positive than that of a cysteine/cystine couple at pH 7.0. Assuming the E'_{cys} to be −0.34 V [26], E'_{c-553} was estimated to be −0.26 V.

Biochemical reactivity of cytochrome c-553

Partially purified cytochrome *c*-553 preparation of purity index 0.15, was shown to be reduced by formate dehydrogenase of *D. vulgaris*, Miyazaki, in the presence of formate [10]. Reducibility of the purified preparation by formate dehydrogenase which was also purified from the same bacterium was thus tested. The results shown in Fig. 2 clearly indicate that the purified cytochrome *c*-553 could be reduced with formate by the action of formate dehydrogenase. The same enzyme preparation failed to reduce cytochrome c_3 in the presence of formate. Cytochrome *c*-553 was not reduced by purified hydrogenase under H_2 nor did it stimulate H_2 evolution from aqueous $Na_2S_2O_4$ in the presence of hydrogenase.

Chemical reactivity of cytochrome c-553

Ferrocytochrome *c*-553 was prepared in an optical cell under N_2 by reducing



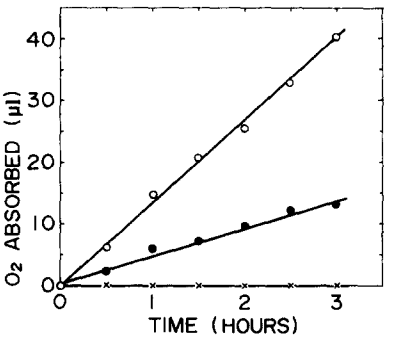


Fig. 4. Equilibration between cysteine/cystine couple and cytochrome *c*-553. Cytochrome *c*-553 solution was added to mixtures of cysteine and cystine in various concentrations in 0.02 M phosphate buffer, and left standing anaerobically in an optical cell. After equilibration, percentages of the ferri and ferro forms of cytochrome were estimated spectrophotometrically, and the pH of the solution was recorded. Temperature was 22°C.

Fig. 5. Oxidation of ascorbate catalyzed by cytochrome *c*-553. Ascorbate oxidase activity of cytochrome *c*-553 was measured by conventional manometric technique. The main compartment of a Warburg vessel contained 21 µmol L-ascorbic acid, 120 µmol Na_2HPO_4 and 60 µmol citric acid in 2.1 ml solution, pH of the solution was adjusted to 6.0 by NaOH. The side arm contained 0.26 µmol cytochrome *c*-553 in 0.9 ml. After preincubation at 30°C under air, the reaction was started by tipping the content of the side arm into the main compartment, and the absorption of O_2 was recorded. ○: complete system, ●: control without cytochrome *c*-553, and X: control without ascorbate.

the ferri form by $Na_2S_2O_4$, and the gas phase was replaced by CO. No spectral change was observed after 20 min incubation.

Ferrocytochrome *c*-553 prepared by formate dehydrogenase and formate in an optical cell under N_2 was treated anaerobically with various redox dyes and the spectrum recorded. It was concluded that no chemical reaction had taken place if a redox dye did not change. By this technique, flavin-adenine dinucleotide, riboflavin phosphate, methylene blue, indigodisulfonate, 5-methylphenazinium methyl sulfate, 1-methoxy-5-methylphenazinium methyl sulfate, methyl viologen and benzyl viologen were shown not to be reduced by ferrocytochrome *c*-553. Ferrocytochrome *c*-553 was oxidized by O_2 under air and also by ferricyanide. Ferricytochrome *c*-553 was reduced by cysteine. It was partially reduced (about 50%) by 10 mM ascorbate, and behaved like ascorbate oxidase: 3 ml 89 μM cytochrome *c*-553 catalyzed the absorption of O_2 at a rate of 15.7 μl/h in the presence of 7 mM ascorbate at pH 6.0. Under similar conditions, the rate of background absorption of O_2 in the absence of cytochrome *c*-553 was 4.0 μl/h (Fig. 5). No direct electron transfer could be observed between cytochrome c_3 and cytochrome *c*-553.

Discussion

D. vulgaris, Miyazaki, contains at least three kinds of *c*-cytochrome, i.e., cytochrome c_3, cytochrome *c*-553 and a high molecular weight cytochrome. More attention has been focused on cytochrome c_3 which is a natural electron acceptor for *Desulfovibrio* hydrogenase, and many publications have appeared on the structure and function of this unusual tetrahemoprotein [1—7]. On the other hand, less attention has been paid to other two cytochromes, probably due to difficulty in purification. In the present work, cytochrome *c*-553 has been purified to homogeneity and was shown to be an electron acceptor for formate dehydrogenase thus confirming previous observations based on rather impure preparations [10].

Le Gall et al. [2,11,12] also described the presence of cytochrome *c*-553 in Hildenborough strain of *D. vulgaris*, and established the amino acid sequence [12]. The molecular weight of their preparation was 8560, a value not very different from that described in this paper. Their cytochrome *c*-553 could be reduced by their formate dehydrogenase preparation [7]. Since their cytochrome c_3 preparation could also be reduced by their formate dehydrogenase [7], no conclusion may be drawn in respect to the acceptor specificity of formate dehydrogenase. The cytochrome *c*-553 reported in this paper was definitely reducible by formate dehydrogenase, whereas cytochrome c_3 was not. Therefore, the acceptor specificity of formate dehydrogenase was conclusively established, and the systemic name for formate dehydrogenase of *D. vulgaris* must be registered as formate:ferricytochrome *c*-553 oxidoreductase in EC subclass 1.2.2.—.

Cytochrome *c*-553 is one of the smallest cytochromes and contains one heme. The amino acid composition described in the present paper is similar to that reported by Bruschi and Le Gall [12], except that the present preparation lacks proline and isoleucine (which were present in their preparation) and contains phenylalanine (which is absent in theirs). It is reduced by ascorbate

(partly) and by cysteine (fully) as seen in the case of their cytochrome *c*-553 preparation [11].

The ferro form of cytochrome *c*-553 is oxidizable by O_2 or by ferricyanide, but in spite of its very negative redox potential (E'_0 = —0.26 V and pH 7.0), it is not oxidized by redox dyes such as methylene blue, indigodisulfonate, 5-methylphenazinium methyl sulfate, 1-methoxy-5-methylphenazinium methyl sulfate and flavin nucleotides, nor does it combine with CO. The millimolar absorbance coefficient of its α-peak (23.9) is lower than cytochrome c_3. Upon alkalinization of the solution the α-peak increases about 1.1-fold. These observations suggested that the heme group of cytochrome *c*-553 is rather inert and protected from the environment.

Le Gall et al. [7] stated that another cytochrome of molecular weight 24500 was present in Hildenborough strain of *D. vulgaris*. In Miyazaki strain of *D. vulgaris*, there is also a larger cytochrome, but its molecular weight is near 60 000, instead. Elucidation of the biochemical functions of these cytochrome components will throw light on the electron transfer systems of both strains of sulfate-reducing bacteria. The present work is a step forward by establishing the function of cytochrome *c*-553 as natural electron acceptor for formate dehydrogenase.

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