PURIFICATION AND PROPERTIES OF THE DIHEME CYTOCHROME (CYTOCHROME *c*-552) FROM *PSEUDOMONAS PERFECTOMARINUS*

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1. Introduction

Many of the ordinarily aerobic pseudomonads are able to grow anaerobically using nitrate and its reduction products as terminal electron acceptors in the biological process termed 'denitrification'. The pseudomonads performing denitrification contain a wide range of *c*-type cytochromes [1]. One of these has been studied in many *Pseudomonas* species and shown to be similar to cytochrome c_4 originally purified [2] from *Azotobacter vinelandii* and further characterized in [3]. The *c*-type cytochrome from pseudomonads [4] is characterized by an α-band absorption maximum at ~552 nm, an oxidation–reduction potential of about +280 mV, and 2 *c*-type heme groups attached to a single 25 000 M_r polypeptide. The N-terminal sequences of cytochromes *c*-552 from 3 different *Pseudomonas* species and the *A. vinelandii* cytochrome c_4 were similar. A heme group was attached near the N-terminus of each of the molecules.

Pseudomonas perfectomarinus (ATCC 14405) is a marine denitrifier [5]. Several *c*-type cytochromes have been detected in fractions prepared from the cell-free extract of *P. perfectomarinus* and appear to have roles in the denitrification process [6,7]. We now report isolation of a *c*-type cytochrome α-band absorption maximum at 551.7 nm) from both the cell wash and the soluble fraction of the cell-free extract. The diheme cytochrome shows several similarities to the *A. vinelandii* cytochrome c_4, which contains 2 heme *c* groups/24 000 M_r [3] and has a mid-point potential of +300 mV [2], and to a number of diheme cytochromes *c* from other denitrifying pseudomonads. We have purified the cytochrome *c*-552 to homogeneity and characterized some of its physical properties. The redox potentials of the heme groups differed by ~350 mV and only the lower potential heme was rapidly auto-oxidizable.

2. Materials and methods

Sephadex G-100, DE-52 and Bio-Gel HTP were obtained from Pharmacia Fine Chemicals, Whatman and BioRad Labs., respectively.

P. perfectomarinus (ATCC 14405) was grown at 25°C in the nitrate-containing medium of [6]. The culture was first incubated aerobically with constant stirring for 30 h and then nitrogen replaced oxygen as the sparging gas to create anaerobic growth conditions. Cells were harvested by centrifugation after 18 h of anaerobic growth and washed or extracted as in section 3. Ultraviolet and visible absorption spectroscopic studies were performed using a Varian DMS 90 recording spectrophotometer. The number of *c*-type heme groups/cytochrome molecule was estimated by the pyridine hemochromogen technique based on a $\epsilon^{\text{reduced}}_{550\text{ nm}}$ of 29.1 $cm^{-1} \cdot \mu mol^{-1} \cdot$ ml heme c^{-1} [5].

Cytochrome *c*-552 M_r was determined by the sedimentation equilibrium method [9]. Analytical polyacrylamide disc-gel electrophoresis was done as in [10]. The minimal M_r was determined by SDS–polyacrylamide gel electrophoresis as in [11].

Salt-free protein samples were hydrolyzed in an evacuated and sealed tube with 6 N HCl for 24, 48, and 72 h and analyzed for amino acid composition with a Beckman Model 120 C automatic amino acid analyzer. Cysteine and cystine were determined as cysteic acid by oxidation with performic acid prior to

acid hydrolysis [12]. Tryptophan was determined after hydrolysis in the presence of 2% thioglycolic acid [13].

Mid-point redox potentials of the *c*-type heme groups of cytochrome *c*-552 from *P. perfectomarinus* were determined spectroelectrochemically using the method in [14].

The modified Biuret method as developed [15] was used for the protein determination with horse-heart cytochrome *c* as the protein standard.

EPR measurements were performed with a Varian E-109 spectrometer essentially as in [16]. EPR spectra were directly transmitted via a Nova 1 (Data General Corp.) minicomputer onto a Ball magnetic disc system for storage of data and subsequent handling of experimental spectra. Other EPR and experimental conditions are under fig.3. The spectra were obtained under non-saturating microwave power conditions.

3. Results and discussion

3.1. *Purification of* P. perfectomarinus *cytochrome c-552*

The cytochrome from both the cell wash (obtained at a low concentration of Tris–HCl buffer) and the soluble fraction of the cell-free extract was purified separately to a homogeneous state by the following purification procedures:

Cell wash: Cells (750 g wet wt) were washed 3 times with 70 ml 0.01 M Tris–HCl buffer (pH 7.6) at 4°C. The supernatant separated after each wash was combined and dialyzed against 0.01 M Tris–HCl buffer (pH 7.6) for 24 h with 3 changes of dialysis buffer. The dialyzed preparation was then applied onto a DE-52 column (4 × 14 cm) pre-equilibrated with 0.01 M Tris–HCl buffer (pH 7.6). A stepwise NaCl gradient in the same buffer was used to elute the protein. Cytochrome *c*-552 was eluted when NaCl reached 0.1 M. The cytochrome fraction was again dialyzed against 0.01 M Tris–HCl buffer (pH 7.6) to remove NaCl and loaded onto another DE-52 column and the cytochrome eluted the same as before. The eluted cytochrome fraction was applied to a hydroxyapatite column (2 × 15 cm) pre-equilibrated with 0.1 M NaCl in 0.01 M Tris–HCl buffer (pH 7.6). The protein was eluted from the column with a stepwise decreasing NaCl gradient in identical buffer until a zero concentration of NaCl was reached and then with increasing concentration of potassium phosphate buffer (pH 7.6). The cytochrome eluted from the column when potassium phosphate buffer reached 50 mM. Another DE-52 column was used in the same way as the first and second DE-52 columns. The cytochrome-rich fraction obtained from this final DE-52 column was concentrated with an Amicon diaflo apparatus and loaded onto a Sephadex G-100 column and eluted with 0.05 M Tris–HCl buffer (pH 7.6). After this step the cytochrome was found to be electrophoretically pure. The process yielded ~50 mg pure cytochrome *c*-552.

Cell-free extract: The washed cells from the above procedure were resuspended in 500 ml 0.05 M Tris–HCl buffer (pH 7.6) and broken by passing through a Mantan-Gaulin homogenizer 3 times at 9000 lb . in^{-2}. A few mg DNase was added to lower the viscosity of the homogenate. Neutralized streptomycin sulfate was then added at 0.5 mg/mg protein and the preparation centrifuged in the cold at 13 200 × *g* for 30 min in a Sorvall centrifuge. The supernatant obtained was termed the cell-free extract. The cell-free extract was next subjected to ultracentrifugation at 100 000 × *g* for 2 h. The supernatant which was now free from particulate matter was decanted and dialyzed against 0.015 M Tris–HCl buffer (pH 7.6) for 24 h with 3 changes of dialysis buffer. The precipitate formed during dialysis was removed by centrifugation. Cytochrome *c*-552 was purified from the resulting preparation following essentially the same steps that were used for the cell wash. The yield of electrophoretically pure cytochrome *c*-552 was 60 mg. No conclusion can be drawn as to the cellular localization of cytochrome *c*-552 as approximately equal amounts were found in the wash and the extract.

3.2. *Spectroscopic properties*

Fig.1 shows the UV-visible absorption spectrum of the purified cytochrome. Ascorbate reduced the cytochrome to a half-reduced state whereas sodium dithionite completely reduced the cytochrome. The absorption maxima and molar extinction coefficients were determined as shown in table 1. When the auto-oxidizability of the reduced cytochrome was tested, the ascorbate-reduced cytochrome was reoxidized extremely slowly even when oxygen was bubbled through the solution. In contrast, the dithionite-reduced cytochrome was rapidly but only partially reoxidized. When the reduction state of the ascor-

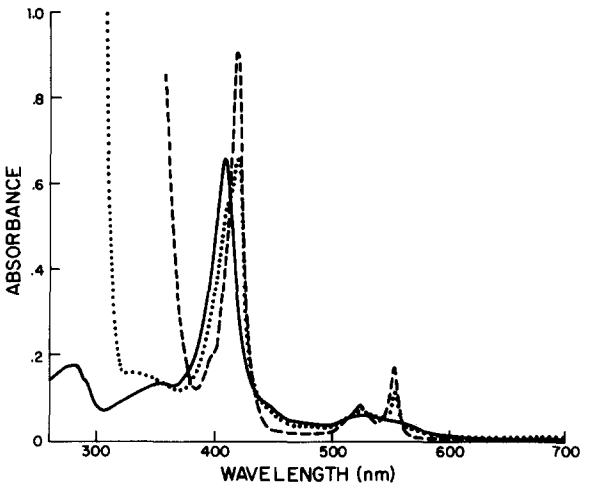

Fig.1. Absorption spectrum of *P. perfectomarinus* cytochrome *c*-552: (——) oxidized; (. . .) ascorbate-reduced; (– – –) dithionite-reduced. Protein was 0.15 mg/ml and the buffer used was 0.1 M sodium phosphate buffer (pH 7.0). It is to be noted that the ascorbate-reduction and dithionite-reduction were allowed to proceed for 20 min and 5 min, respectively, for the system to reach steady state.

bate-reduced level was reached, further reoxidation again proceeded slowly.

3.3. *Physical properties*

3.3.1. Heme prosthetic group

The absorption spectrum of *c*-552 is that of a typical *c*-type heme-containing protein (fig.1). Acid acetone (0.015 N HCl in acetone) did not extract the heme group from the cytochrome. The pyridine hemochromogen also displayed a typical *c*-type heme absorption spectrum with the reduced α-band at 550 nm.

3.3.2. Relative molecular mass

The M_r of the cytochrome determined by the sedimentation equilibrium method was 25 800. SDS–polyacrylamide gel electrophoresis indicated a minimum of 27 900 M_r. It thus appears that the cytochrome *c*-552 is a monomer of 25 800 M_r.

3.3.3. Heme content

The no. heme *c* groups/cytochrome molecule was determined by the pyridine hemochromogen method. It was shown that the cytochrome contained 1.56 heme *c*-groups/25 800 M_r molecule or 1.69 heme *c* groups/27 900 M_r molecule obtained from SDS–polyacrylamide gel electrophoresis. The low values can be due to the fact that horse-heart cytochrome *c* was used as the standard in the protein determination. From the pyridine hemochromogen data and other results (UV-visible absorption spectroscopic and EPR spectroscopic studies), however, it can be concluded that cytochrome *c*-552 contains 2 *c*-type heme groups/protein molecule.

3.3.4. Amino acid composition

The results of amino acid analyses of *c*-552 and the proposed amino acid composition of the *A. vinelandii* cytochrome c_4 [3,7] are shown in table 2. The overall amino acid composition of the cytochrome *c*-552 and that of the *A. vinelandii* cytochrome c_4 shows significant similarities in all groups of amino acids. The number of cysteine residues found (3)

Table 1
Absorption maxima and molar extinction coefficients of *P. perfectomarinus* cytochrome *c*-552[a]

Oxidized		Ascorbate-reduced[b]		Dithionite-reduced[c]	
λ_{max} (nm)	$\epsilon \times 10^{-3}$	λ_{max} (nm)	$\epsilon \times 10^{-3}$	λ_{max} (nm)	$\epsilon \times 10^{-3}$
408.5 ($\gamma_{oxid.}$)	203.90	408.5 ($\gamma_{oxid.}$)	157.74	418.0 ($\gamma_{red.}$)	312.20
349.5 (shoulder)	38.36	418.0 ($\gamma_{red.}$)	207.58	523.0 (β)	24.42
282.0	50.88	523.0 (β)	20.1	551.7 (α)	51.28
		551.7 (α)	33.02		

[a] These values were determined in 0.1 M sodium phosphate buffer (pH 7.0)
[b] The system was incubated for 30 min after the addition of ascorbate to permit the steady reduction state to be reached
[c] The system was incubated for 5 min after the addition of dithionite to permit the steady reduction state to be reached

Table 2
Amino acid analysis (residues/cytochrome molecule) of *P. perfectomarinus* cytochrome *c*-552

Amino acid	6 N HCl hydrolysis			Proposed amino acid composition	Proposed amino acid composition[a] of *A. vinelandii* cytochrome c_4
	24 h	48 h	72 h		
Asp + Asn	26.97	26.43	26.51	27	24
Thr	13.83	13.79	13.78	14	10
Ser	11.36	11.60	11.31	12	12
Glu + Glu	21.17	21.02	21.19	21	22
Pro	10.92	10.63	10.88	11	13
Gly	31.41	31.10	31.28	31	27
Ala	34.54	35.17	35.16	35	37
Val	15.19	15.94	16.07	16	6
Met	6.32	5.49	5.43	7	8
Ile	7.55	7.00	7.01	7	7
Leu	15.37	15.02	14.99	15	16
Tyr	9.27	9.22	9.21	9	6
Phe	8.43	8.37	8.38	8	5
His	4.74	4.84	4.82	5	4
Lys	17.15	17.11	17.11	17	12
Arg	7.00	6.93	6.96	7	8
Cys[b]	2.36	3.01		3	4
Trp[c]	2.69	3.25		3	0
Total				248	221

[a] Obtained from [15]; [b] Determined by hydrolysis after performic acid oxidation; [c] Determined by hydrolysis in the presence of 2% thioglycolic acid

could indicate that 1 heme is bound to the apoprotein by only 1 thioether linkage, as shown for cytochrome *c*-557 from *Crithidia* [18]. The number of histidine and methionine residues is sufficient to allow the histidine–histidine or histidine–methionine ligand arrangements for both heme *c*-groups. However, the absence of the 695 nm absorption maximum in the oxidized light-absorption spectrum (fig.2) indicates that a typical histidine–methionine ligand arrangement does not exist in this cytochrome. Based on the amino acid composition, cytochrome *c*-552 was calculated to be 26 073 M_r which agrees well with the value obtained by the sedimentation equilibrium method.

3.3.5. Mid-point oxidation-reduction potential

Via spectro–electrochemical coulometric and potentiometric titrations [14] the 2 heme *c*-groups of the cytochrome *c*-552 exhibited 2 quite different formal reduction potentials: the high-potential heme had a formal reduction potential of 174 ± 17 mV *vs* NHE and that of the low-potential heme was estimated to be ~−180 mV. The different formal reduction potentials of the 2 heme *c*-groups are consistent with observations obtained by 2 spectroscopic methods, i.e., the light-absorption spectral changes found on reduction of the cytochrome with ascorbate or with dithionite, along with the auto-oxidization properties described previously, and the EPR data below.

3.3.6. EPR spectroscopic properties

EPR studies of ferric cytochrome *c*-552 at 9 K revealed 2 distinct ferric low-spin heme signals arising from 2 non-equivalent heme species (fig.3A). The first ferric low-spin heme species has $g_z = 3.25$ while the other ferric low-spin heme species has $g_z = 2.88$ and $g_y = 2.22$. In the latter case, based on the g_z and g_y values observed, the *c*-type heme group has histidine as its sixth ligand [19,20]. The sixth ligand of the second *c*-type heme group, however, does not fall into an appropriate category [19,20] and does not seem to be the methionine–sulfur since the oxidized

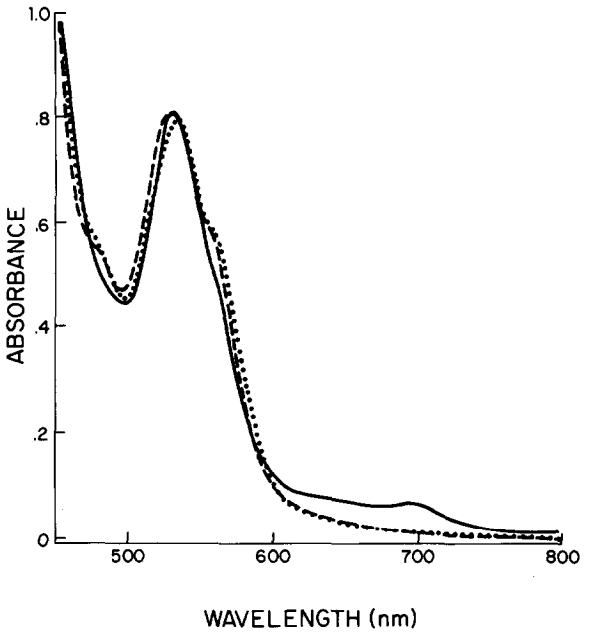

Fig.2. Absorption spectral study on the ligand arrangement of heme iron atoms in *P. perfectomarinus* c-552: (——) horse heart cytochrome *c*; (. . .) *Desulfovibrio vulgaris* cytochrome c_3; (– – –) *P. perfectomarinus* cytochrome *c*-552. Horse heart cytochrome *c* has a ligand arrangement of histidine–methionine type and shows a peak at 695 nm region. *D. vulgaris* cytochrome c_3 has a ligand arrangement of histidine–histidine and does not have the 695 nm peak. The *P. perfectomarinus* cytochrome *c*-552 apparently shows similarity to the *D. vulgaris* cytochrome c_3.

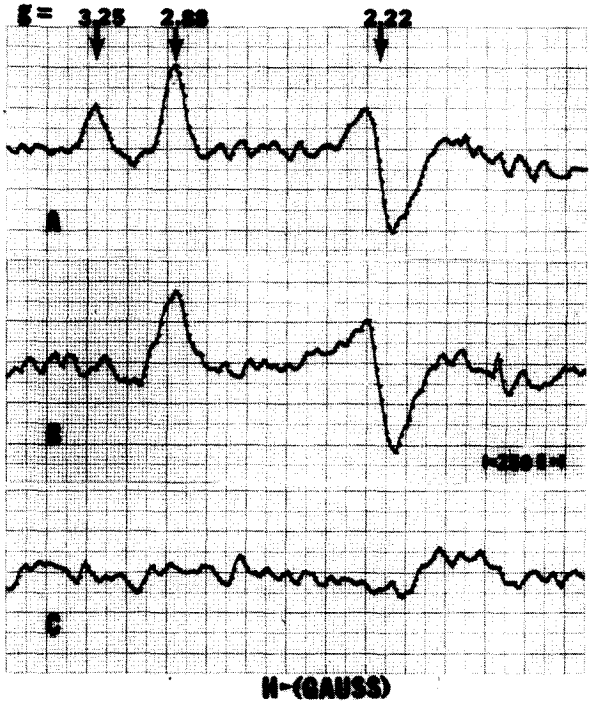

Fig.3. EPR spectra of the oxidation–reduction properties of *P. perfectomarinus* c-552. EPR measurements were made with a Varian model E-109 spectrometer operating at 100 kcycles modulation and an Air Products ADP-E automatic helium temperature control system. The EPR spectrometer was interfaced with a Nova 1 (Data General Corp.) minicomputer for data collection. EPR conditions: microwave power, 0.1 mW; modulation amplitude, 5 G; scanning rate, 1000 G/min; time constant, 0.1 s; temperature, 9 K; frequency, 9.1142 GHz. (A) Oxidized cytochrome *c*-552 (2.36 mg protein/ml in 0.1 M Tris–HCl, pH 7.6); (B) as (A) but reduced with slight excess of sodium ascorbate; (C) as (A) but reduced with slight excess of sodium dithionite.

light absorption spectrum lacks a 695 nm absorption peak.

Fig.3B shows that the low-spin ferric heme resonance at $g_z = 3.25$ disappeared on reduction with ascorbate. The g_z signal at 3.25 may be attributed to the high-potential *c*-type heme with the spectroelectrochemically determined E_m of 174 ± 17 mV while the other g_z signal at 2.88 is tentatively assigned to the low-potential *c*-type heme ($E_m \approx -180$ mV). Fig.3C shows that the latter signal at $g = 2.88$ (and the signal at $g_y = 2.22$) disappeared on reduction with dithionite. The disappearance of the g_z signal at 2.88 is consistent with its assignment to the status of low-potential heme. The disappearance of all signals on dithionite reduction indicates that the ferric signals initially present were reduced to the ferrous state. The 2 distinct low-spin ferric heme EPR signals indicate non-equivalent environments, and may explain the reason why the two hemes have different redox potentials.

Although the physiologicals function of the *P. perfectomarinus* cytochrome *c*-552 remains unclear, it will be interesting to study, especially with respect to the different redox potentials of the 2 *c*-type heme groups. In some *Pseudomonas* species, the cytochrome *c*-552 was suggested to be related to the nitrite reductase system during denitrification [21–25]. In [26], an amino-glycoside-resistant mutant of *P. aeruginosa* defective in both cytochrome *c*-552 and nitrate reductase was reported; however, actual identity of the cytochrome *c*-552 in this case remains to be clarified.

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