Purification and characterization of low potential *c* cytochromes from *Desulfovibrio desulfuricans* membranes

Jeffrey F. Kramer, Daniel H. Pope and J.C. Salerno

Biophysics Group and Biology Department, Rensselaer Polytechnic Institute, Troy, NY 12181, USA

Received 23 July 1986

Desulfovibrio desulfuricans grown in a lactate-sulfate medium produces, in addition to soluble cytochromes, *c*-type cytochromes which appear to be integral membrane proteins. Two cytochromes can be separated, an abundant 15 kDa cytochrome and a 22 kDa cytochrome. Both have optical spectra characteristics of *c*-type cytochromes. The 15 kDa cytochrome shows two $n = 1$ components in potentiometric redox titrations with midpoint potentials at −130 and −270 mV in the membrane; both were slightly lower in detergent-solubilized preparations. We suggest a designation of cytochrome cc_m for this species. Its properties suggest a function as a transmembrane electron carrier between hydrogen and sulfate.

Cytochrome *Membrane protein* *Electron transfer* *Energy transduction* (Desulfovibrio desulfuricans) *Sulfate reduction*

1. INTRODUCTION

Sulfate-reducing bacteria of the genus *Desulfovibrio* contain a number of electron transfer proteins [1–3]. Considerable data concerning these proteins are available, but their role in metabolism remains obscure. Various species and subspecies exhibit an unexpectedly high degree of variability.

Electron transfer and energy conservation in *Desulfovibrio* are poorly understood, although a number of interesting hypotheses have been proposed. Dehydrogenases, reductases and electron carriers involved in dissimilatory sulfate reduction have been localized with respect to the periplasmic space, membrane and cytoplasm [4–7]. Membranes from *Desulfovibrio* have not been as intensively studied, but *b*- and/or *c*-type cytochromes, hydrogenase, fumarate reductase and menaquinone-6 have been reported [5,6,8–13].

Abbreviations: DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PES, phenazine ethosulfate

Here, we report further on integral membrane *c*-type cytochromes from *D. desulfuricans* grown on lactate-sulfate. These are the major electron carriers in these membranes; in other *Desulfovibrio* species *b* cytochromes have been reported with either sulfate or fumarate as the electron acceptor.

2. MATERIALS AND METHODS

D. desulfuricans (ATCC 7757) was grown anaerobically in 20 l glass carboys containing 19 l of Postgate's medium C [14] and which had been stoppered after autoclaving. 1 l of a culture grown in medium C for 24 h was used as inoculum. The cells were harvested after 24 h at 35°C using a continuous flow adaptor for a Sorvall SS-34 rotor.

The starting material was 50 g frozen cell paste. Periplasmic proteins were removed by the method of Van der Westen et al. [15]. Washed cells were resuspended in 100 ml of 50 mM potassium phosphate buffer (KP) and passed twice through a French press at 7000 lb/inch^2. The extract was centrifuged at $10^5 \times g$ for 1 h. The pellet was resuspended in 50 mM KP (pH 7.5), 100 mM KCl

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

and centrifuged again. The resulting pellet was resuspended in 50 mM KP (pH 7.5), 1% Triton X-100, 2% cholate and incubated for 12 h at 4°C. The extract was centrifuged at $10^5 \times g$ for 1 h. The reddish-brown supernatant was concentrated to 150 ml with an Amicon PM 30 filter, dialyzed against 50 mM KP (pH 7.5), 0.5% cholate and loaded onto a 6 × 10 cm column of calcium phosphate [16]. Most of the cytochrome eluted in the nonadsorbed fraction. This was concentrated to 100 ml using an Amicon PM 30 filter and dialyzed against 20 mM KP (pH 7.5), 0.5% cholate, loaded onto a 5 × 40 cm column of DEAE-Sephacel equilibrated with the same buffer, and eluted with a linear gradient of NaCl (0–0.8 M) in 1 l.

Polyacrylamide gels containing a gradient of 7.5–15% acrylamide and 0.1% SDS were done as described by Laemmli [17]. Gels were stained for heme-associated peroxidase activity [18] or with Coomassie blue R-250 [19]. Protein was determined by the method of Lowry et al. [20]. BSA or horse heart cytochrome *c* were used as standards. Pyridine hemochromogen assays were performed as described by Takaichi and Morita [21].

UV-visible absorbance spectra were recorded using a Johnson Foundation DBS-3 scanning dual-wavelength spectrophotometer. Potentiometric titrations were performed as described by Dutton [22]. Mediators used were 2-hydroxy-1,4-naphthoquinone (0.05 mM), pyocyanin (0.0025 mg/ml), safranine O (0.01 mM), PES (0.05 mM), PMS (0.05 mM), benzyl viologen (0.01 mM) and methyl viologen (0.01 mM).

3. RESULTS

The soluble fraction of *D. desulfuricans* grown on lactate and sulfate contains a *c*-type cytochrome [23], hydrogenase [4,24,25], ferredoxins [26] and other components. These could be almost completely removed from the membranes by washes with 100 mM KCl.

The membrane cytochromes could be solubilized in Triton, cholate or deoxycholate. The most complete solubilization was achieved using Triton, alone or with other detergents. Fig.1a shows the optical spectrum of the crude detergent extract. Major features are the Soret band at 415 nm and the 280 nm band caused by aromatic amino acid

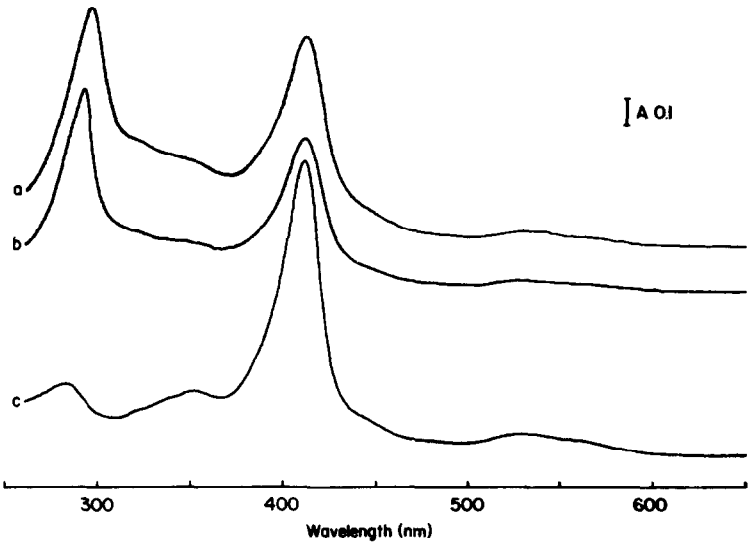

Fig.1. Optical spectra of detergent-solubilized cytochromes at various stages of purification, showing the increasing contribution of ferriheme relative to iron-sulfur and aromatic amino acid chromophores. (a) Crude detergent extract after removal of insoluble material by centrifugation and dialysis against buffer solution with 0.5% cholate. (b) Unadsorbed fractions from calcium phosphate column. (c) Two band fractions eluted from DEAE-Sephacel column at 10 mM NaCl containing 15 kDa cytochrome.

residues. There are broad bands at 300–400 nm contributed by membrane-bound hydrogenase, and broad weak bands representing ferriheme α and β transitions.

As the data of fig.1b imply, the calcium phosphate column removes most of the hydrogenase, which can be collected and further purified. The most extensive purification is achieved in the DEAE-Sephacel step. Fig.1c shows the optical spectrum of the cytochrome which eluted near 10 mM NaCl. The absorbance in the 280 nm region has been greatly reduced relative to the Soret band. A less abundant component, eluted at about 500 mM NaCl, has a similar spectrum except for a higher A near 280 nm (not shown).

The more abundant cytochrome fraction consists mainly of two bands on SDS-PAGE; only the lower molecular mass band had significant heme content. The gels are not shown due to space limitations. The molecular mass of the cytochrome was about 15 kDa; the red color of the heme was visible in the unstained gel and was readily confirmed by staining for heme. The other cytochrome fraction was less pure. It contained several polypeptides including a small amount of the 15 kDa component. The other heme protein had a molecular mass of 22 kDa.

The optical spectrum of the reduced 15 kDa cytochrome is shown in fig.2a. The Soret band is at 420 nm and α- and β-bands can be seen at 525 and 553 nm. Fig.2b,c shows the reduced minus oxidized difference spectra of purified 15 kDa cytochrome and the membrane fraction before solubilization. The spectra are similar, with minor differences attributable to the removal of the other components rather than to large modifications of the heme site during solubilization and purification. CO addition had no effect on the spectra (not shown) at any stage.

Table 1 summarizes the results of the purification procedure. Changes in relative intensities of heme and protein optical bands demonstrate effective purification; concurrently, the SDS-PAGE pattern simplified from 10–12 bands to 2 bands. We estimated that the final yield was about 10%, using the α-band intensities.

Potentiometric redox titrations of membranes before solubilization, monitored at the α-band, showed multiple components as illustrated in fig.3.

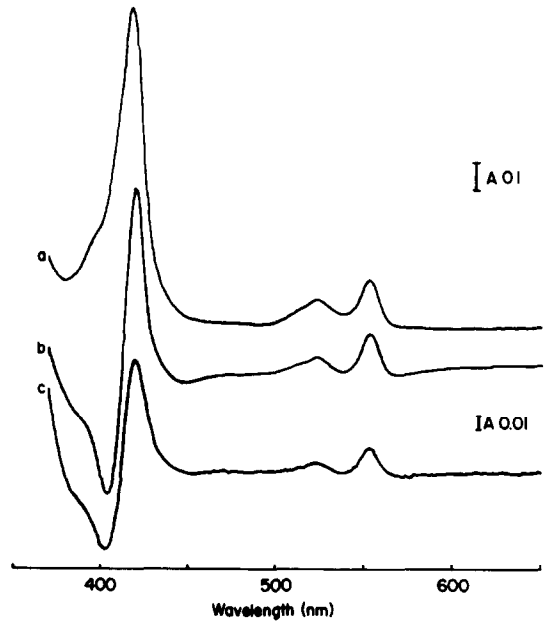

Fig.2. Optical spectra of cytochromes from *D. desulfuricans* membranes showing typical *c*-type cytochrome α-bands in the reduced state. (a) Dithionite reduced spectra of fractions from fig.1c. (b) Reduced minus oxidized difference spectra of fractions from a. (c) Reduced minus oxidized difference spectra of membrane fragments before detergent solubilization (gain × 10). Extinction coefficient of the α-band was calculated as 24 (A/mM per cm) by comparison with pyridine hemochromagen spectra.

Table 1

Purification of cytochrome cc_m

Step	A (280 nm)	A Soret	A (α-)	Total heme (10^{-6} mol)
Membrane fragments	–	14.0	1.2	3.9
Detergent extracts	4.4	3.9	0.67	4.4
$CaPO_4^{2-}$ fractions	3.7	2.8	0.50	2.2
DEAE-Sephacel fractions	2.6	11.0	2.0	0.43

Absorbance of cytochrome cc_m-containing fractions at important stages of purification, indicating increasing heme/protein ratio. Yield in terms of total heme present in the membrane was about 10%, but the actual yield of cytochrome is higher because the membrane also contains other cytochromes, primarily the 22 kDa species

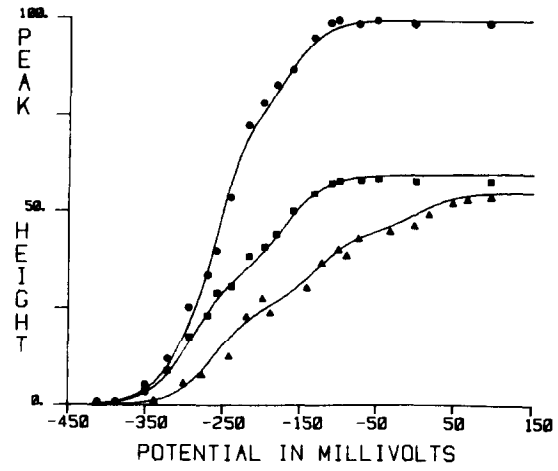

Fig.3. Potentiometric redox titrations of *Desulfovibrio* membrane cytochromes; amplitude of α- or Soret bands in redox difference spectra (fully reduced minus potential given) plotted against E_h. Titrations were carried out in Tricine buffer at pH 8. (▲) α-Band of membrane fragments as in fig.2c, measured at 556 nm. Simulation: three $n = 1$ components, −270, −130 and 10 mV. (■) α-Band of 15 kDa cytochrome as in fig.2b, measured at 553 nm. Simulation: two $n = 1$ components, −300 and −175 mV. (●) Soret band of 15 kDa cytochrome, measured peak to trough. Simulation: two $n = 1$ components at −300 and −175 mV, one $n = 2$ component at −250 mV.

Two components of nearly equal contribution were observed with potentials of about −130 and −270 mV, higher than those reported for soluble cytochromes in *Desulfovibrio* but still relatively low for *c* cytochromes. A minor component was observed near 10 mV but this may be due to overlap from the optical spectra of redox mediators or from additional components in the membrane.

The purified 15 kDa cytochrome titrated as two components with potentials of about −175 and −300 mV. As shown in fig.3, the α-band titration had no 10 mV component, because of the removal of a component in purification or the higher ratio of heme to mediator which can be achieved with the purified system. There is good agreement between titrations monitored at the Soret band and those monitored at the α-band, except that an $n = 2$ species with an E_{m8} of −250 mV was included to fit Soret titrations. This may correspond to 2-hydroxy-1,4-naphthoquinone, one of the mediators. The 22 kDa cytochrome also appears to have a low redox potential, but has not yet been resolved from the other cytochrome.

4. DISCUSSION

Several chemiosmotic models have been proposed for electron transfer and proton translocation in *Desulfovibrio*. A common feature has been the requirement for electron transfer from periplasmic donors (hydrogen and cytochromes *c*) to cytoplasmic terminal oxidants, in this case the couples involved in the eight-electron reduction of sulfate to sulfide.

The potentials of periplasmic components vary from about −300 mV (cytochromes *c*) to −420 mV (H_2 at pH 7). If the sulfate/sulfite couple is primed with ATP via the APS system [27], the four two electron couples from sulfate to sulfite will all have effective midpoint potentials of about −100 mV. The 15 kDa cytochrome appears well suited for a role in electron transfer between periplasmic and cytoplasmic components. Two hemes could be sufficient to catalyze transmembrane electron transfer, as has been proposed for the *b* cytochromes of mitochondrial and bacterial cytochrome bc_1 complexes. The potentials of the two *c* components are suitable for a transmembrane electron transfer component.

Both cytochromes reported here appear to be integral membrane proteins, differing in solubility, electrochemistry and/or size from previously reported cytochromes from this genus. A previously reported particulate cytochrome c_3 has a molecular mass of 26 kDa [28]. We designate the 15 kDa species cytochrome cc_m because of its two components and location. It appears to be an electron-carrying arm of the type proposed by Mitchell [29], although this remains to be proven. The role of such an arm in *Desulfovibrio* is simplest to describe in electron transfer from H_2 to sulfate.

Oxidation of H_2 in the periplasm results in release of external scalar protons. Inward exergonic electron transfer through the *c* cytochrome would establish a transmembrane electrical potential.

We expect that the high-potential heme is located towards the cytoplasmic side. Electrons would be transferred to the terminal oxidases in the cytoplasm, and reduction of sulfate to sulfide

would result in alkalinization of the cytoplasm because of the uptake of internal scalar protons. Thus both a transmembrane electrical potential and pH gradient would be established. This is similar to the model proposed recently for *Thiobacillus ferrooxidans* [30], except that in *Desulfovibrio* electron transfer would be much more energetically favorable since H_2 is a much better reductant than ferrous iron.

With lactate or pyruvate as the donor, electron transfer would be more complex. The dehydrogenases for these substrates are cytoplasmic in orientation, so that a hydrogen carrying mechanism appears necessary. This could be a combined proton pump and electron-carrying arm, but has been proposed to be due to hydrogen cycling [31]. In H_2 cycling, two hydrogenases are required with opposite orientations, and transmembrane H_2 transfer is accomplished by diffusion of dissolved H_2. The results reported here are consistent with hydrogen cycling, but do not support it over other hypotheses. We suggest that the pyruvate/acetate couple probably drives proton translocation by one of these mechanisms, with the return of electrons to the cytoplasm through the cytochrome. The lactate/pyruvate couple, however, is probably not reducing enough to be coupled in this way. It may therefore reduce sulfate directly in the cytoplasm without intermediate vectorial reactions. The pyruvate produced could then be oxidized by a mechanism which leads to ATP production as implied above. We hope to discuss new models for *Desulfovibrio* electron transfer more fully in a later paper.

REFERENCES

[1] LeGall, J. and Forget, N. (1979) Methods Enzymol. 53D, 613–634.
[2] LeGall, J., DerVartanian, D.V. and Peck, H.D. jr (1979) Curr. Top. Bioenerg. 9, 237–265.
[3] Odom, J.M. and Peck, H.D. jr (1984) Annu. Rev. Microbiol. 38, 551–592.
[4] Bell, G.R., LeGall, J. and Peck, H.D. jr (1974) J. Bacteriol. 120, 994–997.
[5] Badziong, W. and Thauer, R.K. (1980) Arch. Microbiol. 125, 167–174.
[6] Odom, J.M. and Peck, H.D. jr (1981) J. Bacteriol. 174, 161–169.
[7] Steenkamp, D.J. and Peck, H.D. jr (1981) J. Biol. Chem. 256, 5450–5458.
[8] Jones, H.E. (1972) Arch. Microbiol. 84, 207–224.
[9] Hatchikian, E.C. and LeGall, J. (1972) Biochim. Biophys. Acta 267, 479–484.
[10] Maroc, J., Azerad, R., Kamen, M.D. and LeGall, J. (1970) Biochim. Biophys. Acta 197, 87–89.
[11] Weber, M.M., Matschiner, J.T. and Peck, H.D. jr (1970) Biochem. Biophys. Res. Commun. 38, 197–204.
[12] Yagi, T. (1970) J. Biochem. 68, 649–657.
[13] Lalla-Maharajh, W.V., Hall, D.O., Cammack, R., Rao, K.K. and LeGall, J. (1983) Biochem. J. 209, 445–454.
[14] Postgate, J.R. (1979) The Sulfate-Reducing Bacteria, Cambridge University Press, Cambridge.
[15] Van der Westen, H.M., Mayhew, S.G. and Veeger, C. (1978) FEBS Lett. 86, 122–126.
[16] King, T.E. (1978) Methods Enzymol. 53D, 181–191.
[17] Laemmli, U.K. (1970) Nature 222, 680–685.
[18] Thomas, P.E., Ryan, D. and Levin, M. (1976) Anal. Biochem. 75, 168–176.
[19] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Takaichi, S. and Morita, S. (1981) J. Biochem. 89, 1513–1519.
[22] Dutton, P.L. (1978) Methods Enzymol. 54, 411–434.
[23] Postgate, J.R. (1956) J. Gen. Microbiol. 14, 545–572.
[24] Haschke, R.H. and Campbell, L.L. (1971) J. Bacteriol. 120, 249–258.
[25] Kruger, H.J., Huynh, B.H., Ljungdahl, P.O., Xavier, A.V., DerVartanian, D.V., Moura, I., Peck, H.D. jr, Teixeira, M., Moura, J.J.G. and LeGall, J. (1982) J. Biol. Chem. 257, 14620–14623.
[26] Bruschi, M., Hatchikian, E.C., LeGall, J., Moura, J.J.G. and Xavier, A.V. (1976) Biochim. Biophys. Acta 449, 275–284.
[27] Peck, H.D. jr (1962) J. Biol. Chem. 237, 198–203.
[28] Bruschi, M., LeGall, J., Hatchikian, C.E. and Dubourdieu, M. (1969) Bull. Soc. Fr. Physiol. Veg. 15, 381–390.
[29] Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research, Bodmin.
[30] Ingledew, W.J. (1982) Biochim. Biophys. Acta 683, 89–117.
[31] Odom, J.M. and Peck, H.D. jr (1981) FEMS Microbiol. Lett. 12, 47–50.