Volume 216, number 1, 140–144 FEB 04725 May 1987

Isolation of succinate dehydrogenase from *Desulfobulbus elongatus*, a propionate oxidizing, sulfate reducing bacterium

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Received 20 March 1987

Succinate dehydrogenase was purified from the particulate fraction of *Desulfobulbus*. The enzyme catalyzed both fumarate reduction and succinate oxidation but the rate of fumarate reduction was 8-times less than that of succinate oxidation. Quantitative analysis showed the presence of 1 mol of covalently bound flavin and 1 mol of cytochrome *b* per mol of succinate dehydrogenase. The enzyme contained three subunits with molecular mass 68.5, 27.5 and 22 kDa. EPR spectroscopy indicated the presence of at least two iron sulfur clusters. 2-Heptyl-4-hydroxy-quinoline-*N*-oxide inhibited the electron-transfer between succinate dehydrogenase and a high redox potential cytochrome c_3 from *Desulfobulbus elongatus*.

Succinate dehydrogenase; EPR; Succinate pathway; Iron-sulfur center; *(Desulfobulbus elongatus)*

1. INTRODUCTION

The interesting characteristic of the sulfate reducing bacteria belonging to the genus *Desulfobulbus* is their ability to oxidize propionate and to ferment, in the absence of sulfate, pyruvate and lactate into a mixture of acetate and propionate [1]. The two species which have been described so far namely *Db. propionicus* [1] and *Db. elongatus* [2] have been shown to possess the same metabolism although their morphology was significantly different [2]. It has been demonstrated that both the formation and the degradation of propionate occur via the succinate pathway and high levels of fumarate reductase and succinate dehydrogenase activities have been found in extracts of *Db. propionicus* [3].

An unidirectional fumarate reductase has recently been isolated from another sulfate reducing bacterium *Desulfovibrio multispirans* which can use fumarate as a respiratory substrate but is unable to oxidize propionate [4].

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Abbreviations: FR, fumarate reductase; SDH, succinate dehydrogenase; DCPIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulfate; HHOQnO, 2-heptyl-4-hydroxy-quinoline-*N*-oxide; MK-5(H_2), menaquinone-5- with saturated isoprenoid side chain; $ClHgPhSO_3H$, 4-chloromeriphenylsulfonate

2. MATERIALS AND METHODS

2.1. *Growth of the organism and preparation of the membrane fraction*

Db. elongatus was grown on lactate medium as described [2]. Cells (450 g wet wt) were mixed with 10 mM Tris-HCl buffer (pH 7.6) to give a 1:3 (w/v) suspension and were broken by passing through a Manton-Gaulin homogenizer two times at 9000 psi. A few milligrams of DNase were added to lessen the viscosity of the crude extract. The preparation was treated with neutralized streptomycin sulfate (0.5 mg/mg protein) and cen-

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00145793/87/$3.50

trifuged at 13000 × *g* for 30 min at 4°C. The supernatant was recentrifuged at 140000 × *g* for 90 min at 4°C. The pellets from the two centrifugations were combined and suspended in a final volume of 560 ml of Tris-HCl buffer (pH 7.6). This preparation was considered as the membrane fraction.

2.2. *Solubilization and isolation of succinate dehydrogenase*

Unless indicated all buffers were adjusted to pH 7.6 and all procedures were carried out at 4°C. Sodium cholate was first added to the membrane fraction at a concentration of 2 g/l. After stirring for 4 h the preparation was centrifuged at 140000 × *g* for 1 h. The pellet was suspended in 460 ml of 20 mM Tris-HCl buffer containing 1% Triton X-100. This preparation was stirred overnight and then centrifuged for 1 h at 140000 × *g*. The pellets were washed with 250 ml of the same extraction buffer and recentrifuged. The two last supernatants which contained most SDH activity (table 1) were combined and called Triton extract. SDH was separated from other components present in the Triton extract through successive DEAE and Ultrogel ACA 34 columns. The recovery of the initial activity present in the membrane fraction was 20% with a 14-fold increase in specific activity. The purified preparation was free of cytochrome *c* but all attempts to remove the cytochrome *b* were unsuccessful.

2.3. *Assays and metal determination*

SDH activity was determined spectrophotometrically by measuring the decrease in absorbance at 600 nm (ϵ = 18 mM^{-1}) caused by the reduction of DCPIP by succinate in the presence of PMS. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.6), 0.05 mM DCPIP, 0.05 mM PMS, 10 mM sodium succinate. The reaction was started by the addition of the enzyme. Fumarate reductase activity was determined spectrophotometrically by measuring in anaerobic cuvettes the decrease in absorbance at 436 nm (ϵ = 10.6 mM^{-1}) caused by the oxidation of reduced FMN by fumarate. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.6), 10 mM sodium fumarate, 0.2 mM FMN. The FMN was reduced by the addition of a few microlitres of 10 mM sodium dithionite solution. The reaction was started by the addition of fumarate. No activity could be detected when methylviologen was used as an electron donor in the presence of hydrogen and hydrogenase as described in [4] for the determination of *D. multispirans* FR activity.

Protein concentrations were determined with the Lowry method [5] using bovine serum albumin as the standard. The carbonate solution was supplemented with 2% SDS to prevent precipitation due to the presence of Triton X-100.

Total iron was determined by plasma emission spectroscopy using a Jarrel-Ash model 750 atomcorp. Nonheme iron was measured colorimetrically with bathophenanthroline [6].

Total flavin and nonacid extractable flavin were determined by the method of Rao et al. [7].

2.4. *Electrophoresis and molecular mass determination*

Subunit composition was determined by SDS-polyacrylamide gel electrophoresis [8] and molecular mass of the undissociated enzyme by sedimentation equilibrium technique.

2.5. *Electron paramagnetic spectroscopy*

EPR measurements were performed with a Varian E-109 spectrometer interfaced with a Hewlett Packard model 9816 microcomputer.

3. RESULTS AND DISCUSSION

3.1. *Localization and activities of SDH*

Db. elongatus SDH was a tightly membrane-bound enzyme as shown by the fact that sodium cholate was not sufficient to extract it. In contrast, Triton X-100 readily solubilized the enzyme with a yield superior to 90%. The purified SDH catalyzed both fumarate reduction and succinate oxidation and, the rate of fumarate reduction was 8-times less than that of succinate oxidation. The same ratio was also observed in the crude extract and in the membrane fraction. The apparent K_m values for fumarate reduction and succinate oxidation were found to be equal to 14.9 and 23.8 μM, respectively. This and also the fact that resting cells of *Db. elongatus* grown on a lactate sulfate medium oxidize propionate and produce propionate from lactate without any lag [9], suggest that the same enzyme catalyzes in vivo both fumarate reduction and succinate oxidation

although it cannot be ruled out that another enzyme was present but undetected. These results also suggest that *Db. elongatus* is different from *Escherichia coli* which has two distinct enzymes, an SDH involved in aerobic respiration which is repressed during anaerobic growth and an FR which participates in the anaerobic respiration with fumarate as the electron acceptor and which is repressed during aerobic growth [10,11].

3.2. *Molecular mass, subunits and stoichiometry*

Sedimentation equilibrium studies indicated a molecular mass of 170 kDa. SDS-polyacrylamide gel electrophoresis revealed three major bands with apparent molecular masses of 68.5, 27.5 and 22 kDa for a total of 118 kDa. The purity of the preparation was estimated to be 90%. The high value of the molecular mass calculated from the sedimentation equilibrium studies can be explained by the binding of Triton X-100 molecules to the enzyme as it was shown in the SDH of *Neurospora crassa* [12]. Assuming a molecular mass of 118 kDa, quantitative analysis showed the presence of 1 mol cytochrome *b*, 1 mol flavin and 8 mol nonheme iron per mol SDH (table 2). The protoheme of the cytochrome *b* was extractable by acetone-HCl and the flavin was not acid extractable and thus covalently bound to the protein. It is to be noted that both *E. coli* and *Vibrio succinogenes* SDH preparations also contain *b*-type hemes [13,14].

Table 1

Solubilization and purification of *Db. elongatus* SDH

Purification steps	Specific activity[a]	Total units
Membrane fraction	0.78	11800
Cholate pellet	0.75	9720
Triton extract	0.80	8860
DEAE Bio-Gel	8.16	3.212
ACA 34	11.12	2450

[a] μmol DCPIP reduced/min × mg protein

Table 2

Quantitative analysis of *Db. elongatus* succinate dehydrogenase

	Iron, heme and flavin content in atoms or mol per 170 kDa protein
Non acid extractable flavin	0.68
Total iron	9.70
Nonheme iron	7.50
Heme *b*	0.91

3.3. *Light absorption*

The light absorption of the purified SDH showed in its oxidized state peaks at 414 and 530 nm due to the cytochrome *b* and a shoulder at around 450 nm due to the flavin (fig.1). Addition of succinate resulted in partial reduction of both cytochrome *b* and flavin as indicated by the shift of the Soret peak to 429 nm, the appearance of the peak at 560 nm and the decrease of absorbance in the 450 nm region. Complete reduction of flavin

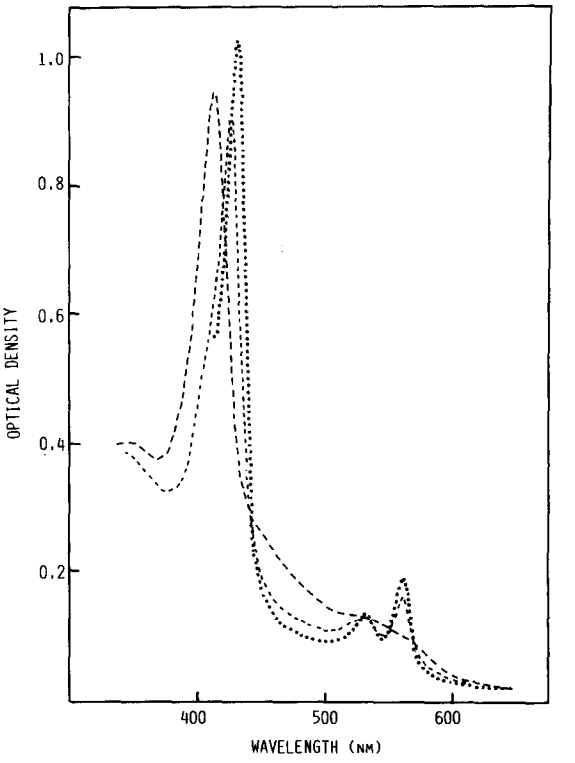

Fig.1. Absorption spectra of *Db. elongatus* succinate dehydrogenase: (– – –) oxidized; (- - -) reduced by 10 mM succinate; (***) reduced by dithionite. The protein concentration was 0.75 mg/ml.

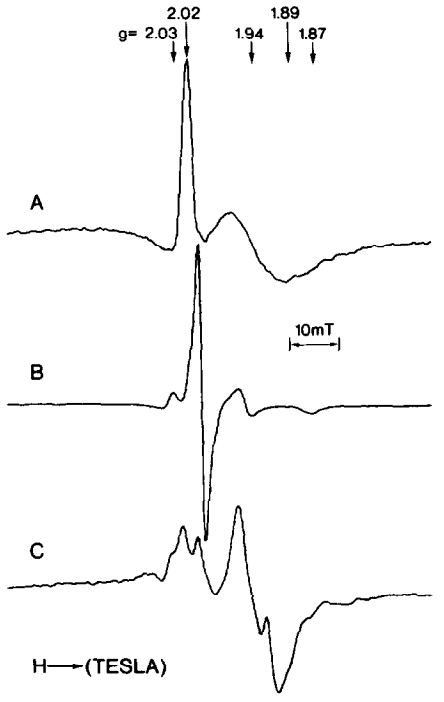

Fig.2. EPR spectra of *Db. elongatus* succinate dehydrogenase (10 mg/ml of protein in 50 mM potassium phosphate buffer, pH 7.6): (A) as prepared, receiver gain 1.25×10^4; (B) as A, but reduced with 10 mM sodium succinate for 10 min, receiver gain 2.5×10^4; (C) as A, but reduced with slight excess of sodium dithionite for 3 min, receiver gain 1.25×10^4. EPR conditions were: temperature, 6 K; microwave frequency, 9.244 GHz; scanning rate, 40 mT/min; time constant, 0.13 s; modulation amplitude, 1 mT; microwave power, 0.02 mW.

and cytochrome *b* was observed after addition of sodium dithionite. In the presence of air, reduced cytochrome *b* was not readily reoxidized.

3.4. *Iron-sulfur clusters and EPR spectroscopy*

SDH in the native state exhibited a complex axial EPR signal with *g* values at 2.02 and 1.93 when measured at 6 K (fig.2). This EPR signal very likely arises from either a [3Fe-XS] or a superoxidized [4Fe-4S] cluster. On reduction with succinate, the initial signal disappeared and was replaced by a weak rhombic EPR signal with *g* values at 2.03, 1.94 and 1.87. Also, a signal appeared at $g = 2.00$ which is attributable to a flavin semiquinone radical species. The subsequent further addition of succinate had no effect on the latter signals. However, when sodium dithionite was added to SDH, the flavin half reduced species at $g = 2.00$ was not detected and a yet more complex rhombic EPR signal was observed with *g* values at 2.05, 2.03, 1.94, 1.93 and 1.87 indicating that a [4Fe-4S] species may have been reduced.

Morningstar et al. [15] observed in *E. coli* fumarate reductase an EPR signal with a sharp peak at $g = 2.02$, a broad trough at 1.97 with an extended tail toward high field and a distinct shoulder at $g = 1.99$ and assigned this signal to an oxidized 3Fe cluster. The spectrum noted in this study (fig.2A) for the native state of succinate dehydrogenase is strikingly similar (with yet a broad shoulder and tail) with the spectrum by Morningstar et al. [15]. They confirmed the nature of the 3Fe cluster by magnetic circular dichroism spectroscopy. By comparison, we tentatively assign the $g = 2.02$ signal in this study to a 3Fe cluster and will await confirmation of this assignment by magnetic circular dichroism and Mössbauer spectral studies in the near future.

3.5. *Inhibition studies*

A tetraheme cytochrome c_3 which has a uniquely high redox potential and which is specifically reduced by succinate in the presence of a membrane preparation, has recently been isolated from *Db. elongatus* [16]. We showed here that HHOQnO inhibited the reduction of this cytochrome c_3 (fig.3) but had no effect on the reduction of ferricyanide. Since *Db. propionicus* was found to contain menaquinone MK-5[H_2] [17], one can assume that it might mediate the electron-transfer from SDH to cytochrome c_3. On the other hand, $ClHgPhSO_3H$ inhibited the reduction of both cytochrome c_3 and ferricyanide, suggesting that it reacts directly on the sulfhydryl groups of the SDH.

3.6. *Physiological significance*

Although *Db. elongatus* SDH and *D. multispirans* are both sulfate reducing bacteria, the properties of their SDH and FR are different. These differences can be interpreted in terms of the physiological significance of the two enzymes. Although in *Db. elongatus* an FR is involved in the propionic fermentation of lactate and pyruvate,

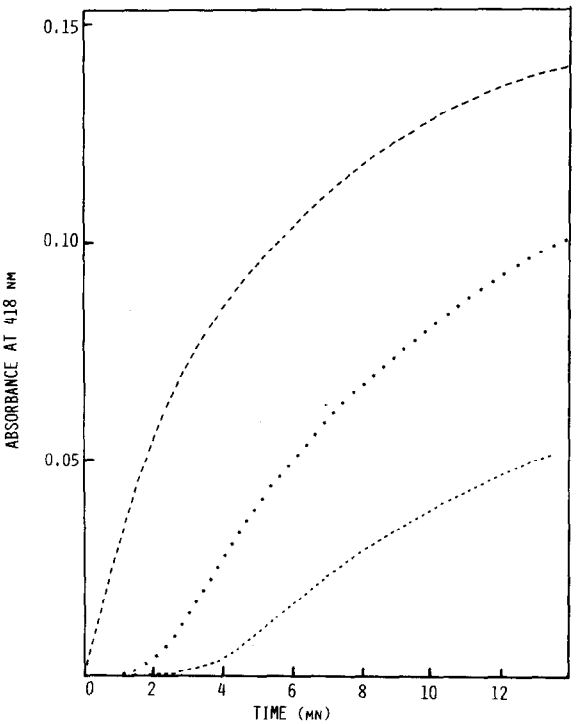

Fig.3. Effect of HHOQnO on the reduction of *Db. elongatus* cytochrome c_3 (1.7 μM) by succinate in presence of the membrane fraction (0.1 mg/ml): (– – –) control without HHOQnO; (***) 9 μM HHOQnO; (- - -) 36 μM HHOQnO. The experiments were performed in anaerobic cuvettes under an atmosphere of oxygen free nitrogen.

this organism does not grow by dismutation of fumarate as do *D. gigas* and *D. multispirans* [18,19]. This can be explained either by the low FR activity of *Db. elongatus* SDH or by the absence in this bacterium of a significant activity of the malic enzyme which catalyzes, in some *Desulfovibrio*, the direct conversion of malate to pyruvate [19] whereas, in *Desulfobulbus*, pyruvate formation from malate is obligatorily associated with the carboxylation of propionyl CoA to methylmalonyl CoA [3]. On the other hand, as *D. gigas* and *D. multispirans* do not grow on propionate or on succinate, the presence of an SDH activity in these organisms is physiologically unnecessary.

ACKNOWLEDGEMENTS

We thank Mrs L. DerVartanian for her excellent technical expertise with the EPR measurements and Mrs P. Kelly and the staff of the fermentation plant of the University of Georgia for growing the cells. This study was supported in part by NSF grant PCM 8213874 to D.V.D., DMB 8602789 to J.L. and AM 01135 and GM 32187 to Dr B.H. Huynh, Department of Physics, Emory University, Atlanta.

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