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EVIDENCE FOR THE PRESENCE OF A *b*-TYPE CYTOCHROME IN THE SULFATE-REDUCING BACTERIUM *DESULFOVIBRIO GIGAS*, AND ITS ROLE IN THE REDUCTION OF FUMARATE BY MOLECULAR HYDROGEN

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SUMMARY

1. The H_2:fumarate reductase activities of particulate fractions prepared from *Desulfovibrio gigas* grown on various media (lactate–sulfate, fumarate or fumarate–sulfate) were measured. Activity was higher in the particulate fraction prepared from cells grown on fumarate or fumarate–sulfate media than in those prepared from cells cultivated on lactate–sulfate.

2. Various inhibitors were tested for their effect on fumarate reduction by H_2 in a particulate fraction of *Desulfovibrio gigas*. A strong inhibitory effect was observed with 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A. These compounds act as inhibitors of an intermediary electron carrier between hydrogenase and fumarate reductase.

3. The strict anaerobe *Desulfovibrio gigas* has been found to contain a *b*-type cytochrome linked to the particulate H_2:fumarate reductase activity. A protoheme obtained by extraction from the particulate fraction was characterized by its reduced pyridine hemochromogen.

INTRODUCTION

Fumarate can be utilized by a number of facultative and obligate anaerobes as the terminal electron acceptor for biological oxidation of various organic compounds and molecular hydrogen[1–7].

It has been observed that some sulfate-reducing bacteria including *Desulfovibrio gigas* and several strains of *Desulfovibrio desulfuricans* and *Desulfovibrio salexigens* grow by fumarate dismutation in a sulfate-free medium[8].

Cell-free preparations of *D. gigas* grown on a lactate–sulfate medium contain all the enzymes necessary for the dismutation of fumarate into acetate and succinate[9,10]. The particulate H_2:fumarate reductase system contains hydrogenase coupled with fumarate reductase by one or more unknown intermediary electron carriers replaceable by viologen dyes. This reaction is of particular importance since it has been

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demonstrated that a particulate fraction of *D. gigas* catalyzes ATP formation during the transfer of electrons from molecular hydrogen to fumarate[11].

In the present paper, we report the increase of the level of fumarate reductase activity in *D. gigas* grown on fumarate or fumarate–sulfate media; the sensitivity of fumarate reductase activity to various inhibitors, especially 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A implied the presence in the particulate fraction of a *b*-type cytochrome which was subsequently detected by low-temperature spectrophotometry. The prosthetic group of this cytochrome *b* was characterized by its reduced pyridine hemochromogen.

MATERIALS AND METHODS

D. gigas was grown at 37 °C. The various media (lactate–sulfate, fumarate or fumarate–sulfate) and methods of preparing extracts as well as particulate and soluble fractions have been already described[9].

A hydrogenase preparation devoid of fumarate reductase activity was made using the method already reported for the solubilization of *D. gigas* cytochrome c'_3 (ref. 12). The harvested cells are carefully suspended in about 2 vol. of 0.05 M phosphate buffer, pH 7.6, then centrifuged for 30 min at 30000 × *g*, and the procedure repeated three times. Under these conditions most of the cytochrome c'_3 and hydrogenase are found in the combined supernatants and there is no appreciable lysis of the cells. It is noteworthy that if the cells are broken prior to that treatment, both cytochrome c'_3 and hydrogenase are found mainly in the particulate fraction and detergents have to be used to solubilize these proteins. The supernatant is used as hydrogenase preparation after removal of cytochrome c'_3 on a small silica gel column.

Protein was estimated by the biuret method[13] and by the method of Lowry *et al.*[14].

Low-temperature spectra were obtained using a Unicam SP 800 spectrophotometer with 1-cm path length cells. The samples were frozen at the temperature of liquid nitrogen and kept at this temperature for a few minutes in a Dewar flask containing liquid nitrogen.

The method used for the extraction and characterization of the heme of the *b*-type cytochrome was similar to that described by Jacobs and Wolin[15]. A particulate fraction from *D. gigas* or *Desulfovibrio vulgaris* strain Hildenborough containing about 200 mg of protein was lyophilized and treated by the different steps indicated in the method. After acid–acetone extraction, the soluble fraction was extracted with acid–ether. The protoheme-containing ether phase was taken to dryness and the pyridine hemochromogen was prepared from the residue[15]. Spectra were obtained with a Cary-14 recording spectrophotometer. The molar extinction coefficient of *b*-hemochromogen is taken to be 34.7 $cm^2/\mu mole$ as indicated by Basford *et al.*[16].

RESULTS

As indicated on Table I, an increased level of fumarate reductase activity is observed in the particulate fraction prepared from *D. gigas* grown on fumarate or fumarate–sulfate media compared to that from cells cultivated on lactate–sulfate. The specific activity of the particulate fraction from *D. gigas* grown on fumarate

TABLE I

FUMARATE REDUCTASE ACTIVITIES OF PARTICULATE FRACTION FROM *D. gigas* GROWN ON VARIOUS MEDIA

Specific activity is expressed as μmoles of hydrogen utilized in 10 min per mg protein. Fumarate reductase activity of the particulate fraction was determined using the Warburg respirometric method. Hydrogen uptake during fumarate reduction was measured in the presence of excess of hydrogenase. Each flask contained: (a) 100 μmoles of Tris–HCl buffer pH 7.6, 20 μmoles fumarate, hydrogenase preparation (150 μg protein), particulate fraction 3.8 mg (1) or 1.4 mg (2) and (3), and 0.05 ml 20% NaOH in center well; total volume, 3 ml; temperature, 37 °C; gas phase, hydrogen. Fumarate in the sidearm was tipped after 20 min equilibration to start the reaction. (b) Same conditions with methyl viologen, 7.5 μmoles, particulate fraction, 1.2 mg (1) or 0.35 mg (2) and (3).

Particulate fraction from D. gigas	*Specific activity*	
	Without methyl viologen (a)	*Plus methyl viologen (b)*
(1) Grown on lactate–sulfate	0.7	3.8
(2) Grown on fumarate	3.21	16
(3) Grown on fumarate–sulfate	3.10	15.4

(or fumarate–sulfate) is almost five times higher than that of the particulate preparation from *D. gigas* grown on lactate–sulfate.

As already described[6,10], methyl viologen enhances fumarate reductase activity of the particulate fraction by substituting for the natural intermediary electron carriers that couple hydrogenase with fumarate reductase. Fumarate reductase is a constitutive enzyme, but the results obtained (Table I) may indicate an increase of fumarate reductase biosynthesis in the presence of fumarate.

It is important to note here that among the sulfate-reducing bacteria, some strains of *D. vulgaris* and *D. desulfuricans* are unable to grow on fumarate media[8]. It was observed, in particular, that extracts of *D. vulgaris* strain Hildenborough were almost devoid of fumarate reductase activity.

TABLE II

EFFECT OF INHIBITORS ON FUMARATE REDUCTASE ACTIVITY

The standard assay described in Table I was used without methyl viologen; inhibitors were added as indicated. The particulate preparation (1.4 mg protein) from *D. gigas* grown on fumarate-sulfate was used for these assays.

Inhibitor	*Final concn (M)*	% *Inhibition*
2-Heptyl-4-hydroxyquinoline-*N*-oxide	$8.3 \cdot 10^{-6}$	80
	$1.6 \cdot 10^{-6}$	52
	$8.3 \cdot 10^{-7}$	32
	$1.6 \cdot 10^{-5}$ *	52 *
	$1.6 \cdot 10^{-6}$ *	19 *
Antimycin A	$5 \cdot 10^{-5}$	51
Amytal	$1 \cdot 10^{-3}$	0
Quinacrine–HCl	$1 \cdot 10^{-3}$	12
Rotenone	$5 \cdot 10^{-5}$	18
KCN	$1 \cdot 10^{-3}$	0
NaN_3	$1 \cdot 10^{-3}$	0

* In this case, the particulate fraction (3.2 mg protein) from *D. gigas* grown on lactate–sulfate was used.

A number of compounds were tested for their inhibitory effect on the fumarate reductase activity of the particulate fraction of *D. gigas* (Table II). These compounds were previously tested on the partially purified hydrogenase from *D. gigas* at higher concentrations than those indicated on Table II and showed no inhibitory activity. No inhibition was observed on fumarate reductase activity with amytal, cyanide and azide whereas rotenone and quinacrine–HCl exhibited a small inhibitory activity. 2-Heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A strongly inhibited fumarate reduction at low concentration (10^{-6} and 10^{-5} M); however, in the presence of methyl viologen, they had no effect. These results show that 2-heptyl-4-hydroxyquinoline-*N*-oxide or antimycin A inhibit the electron flow between hydrogenase and fumarate reductase at the site of an intermediary electron carrier. Since 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A are known to be inhibitors of electron transfer at the site of cytochrome *b*[7,16–21]. it seemed reasonable to look for such a cytochrome in *D. gigas*.

A spectrophotometric study at liquid-nitrogen temperature of *D. gigas* extracts was undertaken to investigate this possibility. The spectra of particulate fractions from *D. gigas* grown on different media are shown on Fig. 1. The first spectrum (particulate fraction from cells grown on lactate–sulfate) exhibits the peaks of a c_3-type cytochrome[12] (with α and β peaks respectively at 550 and 522 nm at low temperature corresponding to the peaks at 553 and 525 nm at room temperature). The peaks of this c_3-type cytochrome are present in all other spectra. Moreover, the spectrum (1) shows shoulders at about 557, 528 and 425 nm, indicating the presence of a *b*-

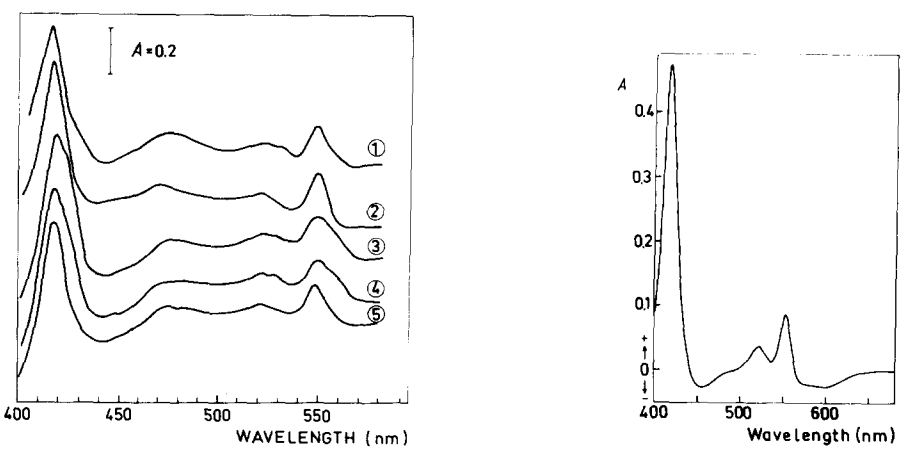

Fig. 1. Reduced cytochrome spectra of particulate and soluble fractions from *D. gigas* and *D. vulgaris* at liquid-nitrogen temperature. The cell contained: Tris–HCl buffer, 50 μmoles, protein concentration, 1.8 mg/ml of the particulate fraction from *D. gigas* grown on lactate–sulfate (1), 3.8 mg/ml of the soluble fraction from *D. gigas* grown on lactate–sulfate (2), 1.6 mg/ml of the particulate fraction from *D. gigas* grown on fumarate (3), 1.5 mg/ml of a particulate fraction from *D. gigas* (grown on fumarate) consisting of the reddish brown gelatinous layer situated above the pellet obtained by centrifugation of the crude extract at 140000 $\times$ *g* for 2 h (4), 1.6 mg/ml for the particulate fraction from *D. vulgaris* strain Hildenborough grown on lactate–sulfate (5); total volume, 2.5 ml. The samples were reduced with sodium dithionite before freezing.

Fig. 2. Difference spectrum of pyridine hemochromogen of the acid–acetone-extractable and acid–ether-soluble pigment extracted from 200 mg of particulate protein of fumarate grown *D. gigas*. The hemochromogen was reduced with a few crystals of dithionite.

type cytochrome. The soluble protein fraction is devoid of cytochrome *b* (spectrum 2).

Spectra 3 and 4 show that when *D. gigas* is grown on fumarate instead of lactate, the proportion of cytochrome *b* to cytochrome c'_3 becomes greater and this increase corresponds to the increase of fumarate reductase activity (Table I). However, it is not known whether this phenomenon is due to an increase in the biosynthesis of cytochrome *b* or to a decrease in the cytochrome c_3' content of the particulate fraction. In *D. vulgaris* strain Hildenborough, cytochrome *b* is not detectable in the particulate fraction, in agreement with the low fumarate reductase activity in this organism. The spectra of the soluble protein fraction from *D. gigas* grown on fumarate and from *D. vulgaris* (not indicated on Fig. 1) are similar to those obtained with *D. gigas* grown on lactate–sulfate.

In order to extract protoheme, the typical ether-soluble prosthetic group of cytochrome *b*, the particulate fraction from fumarate grown *D. gigas* was extracted with acid–acetone by the procedure already described. This procedure does not extract heme of the type associated with cytochrome *c*[15]. The spectrum of the pyridine hemochromogen of the acid–acetone-extractable and acid–ether-soluble heme is shown in Fig. 2. The reduced pyridine hemochromogen exhibits peaks at 556, 523 and 419 nm. These results indicate that the prosthetic group of this pigment is protoheme[22,23], thus confirming the presence of a *b*-type cytochrome in *D. gigas*.

The amount of protoheme extracted from *D. gigas* grown on fumarate was found to be 0.18 nmole/mg of particulate protein. This is twice as high as that found in particulate protein of lactate–sulfate-grown *D. gigas*. Small amounts of protoheme were also extractable from a particulate fraction of *D. vulgaris* strain Hildenborough.

DISCUSSION

The inhibitory action of 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A on fumarate reductase activity as well as the low-temperature spectrophotometric studies and extraction of protoheme from the particulate fraction indicate that a *b*-type cytochrome is present in the strict anaerobe *D. gigas*. This hemoprotein is linked to the particulate fumarate reductase activity and acts as an intermediary electron carrier between hydrogenase and fumarate reductase. Recently, a *b* cytochrome has been found by Jones[24] in *Desulfovibrio africanus*. The presence of a *b*-type cytochrome in the genus *Desulfovibrio* deserves comment. According to Postgate and Campbell[25], the type of cytochrome present in the sulfate-reducing bacteria is a criterion for their classification: only the spore-forming genus *Desulfotomaculum* has been found to contain a *b*-type cytochrome, whereas cytochrome c_3 was the only cytochrome found in *Desulfovibrio*. Our results and those of Jones suggest that the presence of *b* cytochrome is not limited to the genus *Desulfotomaculum*.

A cytochrome *b* linked to fumarate reduction has been already described by Jacobs and Wolin[15] in the strict anaerobe *Vibrio succinogenes*. On the other hand a *b*-type cytochrome linked to the particulate fumarate reductase activity was found in the facultative anaerobe *Proteus rettgeri*[26] and also in the strict aerobes *Bacillus megaterium*[18] and *Mycobacterium phlei*[7]. However, it is to be noted that the strict anaerobe *Streptoccocus faecalis*, which exhibits NADH:fumarate reductase activity, is devoid of cytochromes[19,27].

In the study reported here, the strict anaerobe *Desulfovibrio gigas* has been

found to contain a *b*-type cytochrome linked to the particulate H_2:fumarate reductase activity. The presence of small amounts of *b*-type cytochrome in *D. vulgaris* strain Hildenborough could be associated with lactate dehydrogenase[28]. The nature of the active center of this enzyme is still unknown in *Desulfovibrio* but in at least one other organism a *b*-type heme is present[29].

As the oxidation of hydrogen with fumarate as electron acceptor is coupled with phosphorylation in the particulate fraction from *D. gigas*[11], the presence of a cytochrome *b* in this microorganism suggests that it plays a role in energy-linked reactions. The complete purification and characterization of this *b*-type cytochrome and its relation with the other electron carriers and, in particular, with the quinones already detected in this organism[30,31] are now in progress.

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