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LOW-POTENTIAL CYTOCHROME *b* AS AN ESSENTIAL ELECTRON-TRANSPORT COMPONENT OF MENAQUINONE REDUCTION BY FORMATE IN *VIBRIO SUCCINOGENES*

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Incorporation of the electron-transport enzymes of *Vibrio succinogenes* into liposomes was used to investigate the question of whether, in this organism, a cytochrome *b* is involved in electron transport from formate to fumarate on the formate side of menaquinone. (1) Formate dehydrogenase lacking cytochrome *b* was prepared by splitting the cytochrome from the formate dehydrogenase complex. The enzyme consisted of two different subunits (M_r 110000 and 20000), catalyzed the reduction of 2,3-dimethyl-1,4-naphthoquinone by formate, and could be incorporated into liposomes. (2) The modified enzyme did not restore electron transport from formate to fumarate when incorporated into liposomes together with vitamin K-1 (instead of menaquinone) and fumarate reductase complex. In contrast, restoration was observed in liposomes that contained formate dehydrogenase with cytochrome *b* ($E_m = -224$ mV), in addition to the subunits mentioned above (formate dehydrogenase complex). (3) In the liposomes containing formate dehydrogenase complex and fumarate reductase complex, the response of the cytochrome *b* of the formate dehydrogenase complex was consistent with its interaction on the formate side of menaquinone in a linear sequence of the components. The low-potential cytochrome *b* associated with fumarate reductase complex was not reducible by formate under any condition. It is concluded that the low-potential cytochrome *b* of the formate dehydrogenase complex is an essential component in the electron transport from formate to menaquinone. The low-potential cytochrome *b* of the fumarate reductase complex could not replace the former cytochrome in restoring electron-transport activity.

Introduction

It was earlier proposed that the membrane of *Vibrio succinogenes* contains two cytochromes *b*, with midpoint potentials of −200 and −20 mV [1]. The content of each cytochrome is approximately equal to that of fumarate reductase (0.7 μmol/g protein) [1,2], while that of formate dehydrogenase is smaller (0.1 μmol/g protein) [3]. Cytochrome *b* (−200 mV) specifically binds 1 mol NQNO/mol. As a consequence, the electron transport from formate to fumarate is blocked [2,3]. The redox response in the membrane suggested that the low-potential cytochrome *b* interacts on the formate side and the high-potential cytochrome *b* on the fumarate side of menaquinone in the electron-transport chain [1,2]:

$$\underbrace{\text{FDH} \rightarrow \text{cyt.}b(-200\text{ mV})}_{\text{FDH complex}} \rightarrow \text{menaquinone}$$

$$\rightarrow \underbrace{\text{Cyt.}b(-20\text{ mV}) \rightarrow \text{FR}}_{\text{FR complex}}$$

Abbreviations: DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, reduced DMN; NQNO, 2-(*n*-nonyl)-4-hydroxyquinoline *N*-oxide; E_m, midpoint potential.

In agreement with this scheme, the high-potential cytochrome *b* was found to be an essential component of the fumarate reductase (FR) complex which catalyzes the electron transport from reduced menaquinone to fumarate [4,5]. Furthermore, the formate dehydrogenase (FDH) complex was found to contain cytochrome *b* which binds NQNO [6]. On the other hand, the isolated fumarate reductase complex contained also a low-potential cytochrome *b* in an amount that was equivalent to the enzyme [4]. This suggested that most, if not all, of the low-potential cytochrome *b* interacts on the fumarate side of menaquinone.

We have addressed this problem by preparing a formate dehydrogenase which is devoid of cytochrome *b*. The modified enzyme was incorporated into liposomes together with vitamin K-1 (instead of menaquinone) and fumarate reductase complex in order to find out whether the low-potential cytochrome *b* of the formate dehydrogenase complex is essential for reconstitution of a functional electron-transport chain. An alternative possibility was that the cytochromes of the fumarate reductase complex might be sufficient for reconstitution.

Methods

Preparative procedures

Formate dehydrogenase complex. The first two steps of the preparation of the complex were performed essentially as described earlier [3], except for the following modifications. Dithionite and 4-aminobenzamidine, which were present in the buffers used earlier, were omitted. All buffers in this investigation contained 1 mM dithiothreitol.

(Step 1) The extraction with Tween 80 of the sediment obtained by centrifugation of the lysed bacteria was done only once.

(Step 2) Elution of the enzyme from the hydroxyapatite column was done with a linear phosphate gradient ranging between 30 and 350 mM potassium phosphate. Concentration of the enzyme by pressure dialysis using a Diaflo Ultrafilter XM 100 (Amicon, Lexington, MA, U.S.A.) was 10-fold.

(Step 3) Anion-exchange chromatography was done on DEAE-Sepharose CL-6B. The column (40 ml, inner diameter 1.6 cm) was equilibrated with an anaerobic buffer containing 0.05% Triton X-100, 20 mM Tris, 1 mM NaN_3 and 1 mM dithiothreitol, pH 7.7, 0°C. After loading of 120 mg protein, the column was rinsed with 50 ml of the same buffer. The enzyme was eluted with 200 ml of a linear NaCl gradient (0–0.15 M) in this buffer. Concentration (5–10-fold) was done as in step 2.

Formate dehydrogenase lacking cytochrome b. Guanidinium chloride (1.5 M) was added to the formate dehydrogenase complex (3 g protein/l) as obtained from step 3 of the preparation procedure. This solution (2.5 ml) was layered on 37 ml of an anaerobic linear sucrose gradient (15–27% w/v), pH 7.7, 0°C, that contained 0.05% Triton X-100, 20 mM Tris, 1 mM NaN_3 and 1 mM dithiothreitol, and centrifuged for 6 h at 206 000 × *g* in a VTi 50 rotor (Beckman Instruments). After fractionation of the centrifuge tube contents, the enzymes (formate dehydrogenase lacking cytochrome *b* and formate dehydrogenase complex) were detected by their activity.

Preparation of proteoliposomes by the dilution method. The formate dehydrogenase complex and the formate dehydrogenase lacking cytochrome *b* for this experiment (Table II) were obtained by density gradient centrifugation after treatment with guanidinium chloride (see above). However, the centrifugation was done with 30 mM octylglucoside present instead of Triton X-100.

Liposomes were prepared from 40 mg phosphatidylcholine (Sigma No. P3644) and 0.4 μmol vitamin K-1, which were dissolved in a mixture of $CHCl_3$ and CH_3OH (2 : 1, v/v) at room temperature. N_2 was then blown onto the surface of this solution (4 ml total volume) until the solvents were evaporated. After the addition of 0.8 ml of an anaerobic buffer containing 50 mM potassium phosphate, 1 mM malonate, 1 mM NaN_3 and 1 mM dithiothreitol, pH 7.7, 0°C, the residue was sonicated for 10 min with pulses of 5-s duration and 5-s interruption.

To 0.2 ml of the liposomal suspension, fumarate reductase complex (0.15 mg protein) and either formate dehydrogenase complex or formate dehydrogenase lacking cytochrome *b* (50 μg) were added. The mixture was incubated for 15 min at 0°C, diluted by 20 ml of the anaerobic buffer described above, frozen in liquid N_2 for 10 min and then

thawed. Freeze-thawing was repeated twice, before the proteoliposomes were sedimented by centrifugation (40 min at 150000 × *g*) and suspended in 0.7 ml of the same buffer.

Preparation of proteoliposomes by the dialysis method. The formate dehydrogenase complex (see above) and the fumarate reductase complex [4] used were prepared as described. Incorporation of the enzymes into liposomes (experiment of Table III) was done as described earlier [6,7].

Analytical methods

Formate dehydrogenase activity was measured photometrically by following the reduction of benzyl viologen or DMN [3]. Fumarate reductase was recorded as the activity of $DMNH_2$ oxidation [4]. Fumarate reduction by formate was measured by following the absorbance of fumarate [2]. One unit of activity (U) represents the oxidation of 1 μmol formate or the reduction of 1 μmol fumarate per min at 37°C.

Cytochrome *b* was determined from the absorbance difference between reduced and oxidized forms at 565–575 nm [1].

Protein was measured by counting the radioactivity which had been incorporated when the bacteria were grown in the presence of [^{3}H]leucine [3,4]. The specific radioactivity of the protein was determined by using the biuret method with KCN [3,8].

The peptide composition of the enzymes was determined using gel electrophoresis in the presence of dodecyl sulfate [3]. The protein in the gels was stained with Coomassie blue G-250, and the stain recorded photometrically [3]. The relative amounts of the peptides were obtained from the areas of the bands of the photometric scan by using the colour index ratio 1 : 0.5 : 1 for the M_r 110000, 25000 and 20000 peptides [4]. The molar amounts of the peptides were then calculated from the relative amounts using their molecular weights and the amount of total protein.

Results

Cleavage of formate dehydrogenase complex

The preparation of the formate dehydrogenase complex which was used earlier for reconstitution of the electron transport in liposomes [6] consisted of three different subunits (M_r 110000, 25000 and 20000). For elucidation of the functions of the

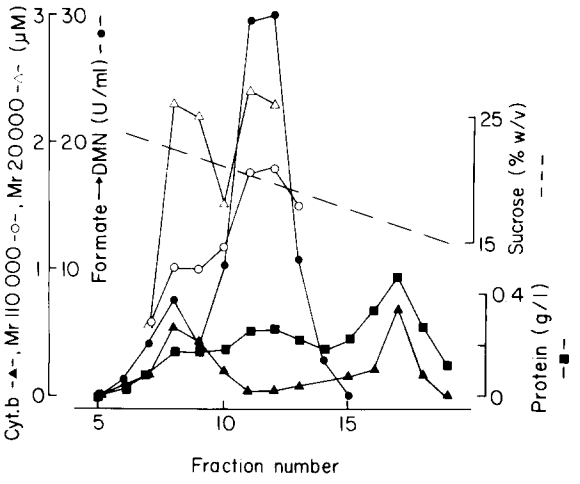

Fig. 1. Sucrose density gradient centrifugation of the formate dehydrogenase complex in the presence of Triton X-100 after treatment with guanidinium chloride. The centrifugation was performed and the measurements were done as described in Methods. The preparation catalyzed DMN reduction by formate with a specific activity of 31 U/mg protein, and contained 3.8 μmol cytochrome *b*/g protein. The recovery of enzyme activity and cytochrome *b* was 115 and 50%, respectively.

TABLE I

PROPERTIES OF THE TWO ENZYME SPECIES OBTAINED BY SUCROSE DENSITY GRADIENT CENTRIFUGATION AFTER TREATMENT OF FORMATE DEHYDROGENASE COMPLEX WITH GUANIDINIUM CHLORIDE

The preparations were obtained from the experiment of Fig. 1. The turnover numbers were obtained from the specific activities and the molecular weights 166000 (fraction No. 8) and 136000 (fraction Nos. 11 + 12) which were calculated from the subunit composition.

Property	Fraction No. 8	Fraction Nos. 11 + 12
Composition	(μmol/g protein)	
M_r 110000 subunit	6	7
M_r 25000 subunit	5.6	< 0.2
Cytochrome *b*	3.1	< 0.1
M_r 20000 subunit	13	9
Turnover number	(min^{-1}) (×10^3)	
Formate → benzyl viologen	40	43
Formate → DMN	7.5	16

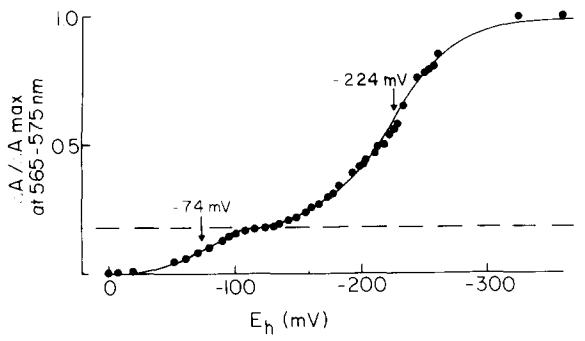

Fig. 2. Redox titration of the cytochrome *b* of the formate dehydrogenase complex. The absorbance at 565–575 nm was recorded as a function of the redox potential E_h (referred to the normal hydrogen electrode) of the solution as described earlier [1]. However, pyocyanine was replaced by 2 μM safranine T. The curves represent theoretical fits to the experimental points according to the Nernst equation and imply that only two cytochromes are present with the given midpoint potentials (E_m) and the relative contributions indicated by the dashed line.

individual peptides, cleavage of the complex and isolation of the fragments were attempted. A preparation of the formate dehydrogenase complex, 50% of the protein of which consisted of the peptides designated above, was treated with guanidinium chloride and subjected to sucrose density gradient centrifugation. The contents of the centrifuge tube were fractionated, and the fractions analyzed for enzymic activity, cytochrome *b* and protein (Fig. 1). The molar concentrations of the M_r 110000 and 20000 peptides in the fractions were calculated from the protein staining of the corresponding bands obtained after gel electrophoresis in the presence of dodecyl sulfate.

The activity of formate oxidation by DMN appeared in two bands. The faster migrating species contained cytochrome *b* and the M_r 25000 peptide while that exhibiting the smaller sedimentation coefficient did not. This result suggests that the M_r 25000 subunit represents cytochrome *b*. The enzyme containing cytochrome *b* represents the formate dehydrogenase complex. It consisted of 1 mol M_r 110000, 1 mol M_r 25000 and 2 mol M_r 20000 peptide in this experiment (Table I). The content of cytochrome *b* was 0.55 mol/mol M_r 25000 peptide. In other preparations this ratio was close to unity. The contents of cytochrome *b* per mol M_r 110000 peptide ranged between 0.5 and 1.5 and those of the M_r 20000 peptide between 1 and 2.5 in these preparations. Redox titration of the formate dehydrogenase complex indicated that most of the cytochrome *b* (82%) is characterized by a midpoint potential of −224 mV (Fig. 2). The residual part (midpoint potential −74 mV) may represent a breakdown product. The enzyme lacking cytochrome *b* was made up of 1 mol M_r 110000 and 1.3 mol M_r 20000 peptide. The turnover numbers of the M_r 110000 subunits of the two enzymes with benzyl viologen as acceptor were nearly identical. The turnover number with DMN as acceptor of the enzyme lacking cytochrome *b* was approx. 2-times greater than that of the complex. It is concluded that the activity of DMN reduction by formate is independent of the presence of cytochrome *b*.

Incorporation into liposomes

To find out whether the cytochrome *b* of the formate dehydrogenase complex is required for the electron transport from formate to fumarate, the enzyme lacking cytochrome *b* was incorporated into liposomes together with vitamin K-1 and fumarate reductase complex. The dialysis technique used earlier for this purpose proved to be unsuitable, because the enzymic activity of the formate dehydrogenase was completely lost. Using the dilution method, however, part of the enzyme activity as measured with DMN was recovered.

Fumarate reductase complex and either formate dehydrogenase complex or formate dehydrogenase lacking cytochrome *b* were added to a mixture of phosphatidylcholine, vitamin K-1 and octylglucoside. The mixture was diluted 20-fold and subjected to freeze-thawing. The resulting proteoliposomes were sedimented by centrifugation and separated from protein that had not been incorporated, using gel filtration. In separate experiments using the individual enzymes, it was found that more than 80% of the protein of the formate dehydrogenase complex and the fumarate reductase complexes and 40% of the formate dehydrogenase lacking cytochrome *b* were incorporated into the liposomes by this procedure (not shown).

The specific activities (based on the total protein in the liposomes) of electron transport and of the individual enzymes of the proteoliposomes

TABLE II

ENZYMIC ACTIVITIES OF PROTEOLIPOSOMES PREPARED BY THE DILUTION METHOD

The two species of the formate dehydrogenase were obtained from sucrose gradient centrifugation in the presence of octylglucoside after treatment of the original complex with guanidinium chloride. Enzyme activities are expressed as U/mg protein.

	Formate → DMN	$DMNH_2$ → fumarate	Formate → fumarate
With formate dehydrogenase lacking cytochrome *b*			
in proteoliposomes	2	25	< 0.1
before incorporation	19	38 [a]	–
With formate dehydrogenase complex containing cytochrome *b*			
in proteoliposomes	2.5	26	2.5
before incorporation	17	38 [a]	–

[a] Activities measured with Triton X-100.

prepared with formate dehydrogenase lacking cytochrome *b* are compared to those of a preparation obtained with the formate dehydrogenase complex in Table II. The activities in the proteoliposomes are also contrasted with those measured in the corresponding mixture of the enzymes before proteoliposome formation. In both types of proteoliposomes nearly 70% of the activity of the fumarate reductase complex ($DMNH_2$ → fumarate) was recovered. The specific activity of formate-DMN reduction (based on total protein) in the proteoliposomes prepared with formate dehydrogenase lacking cytochrome *b* was 11% of that measured before incorporation of the enzyme. After correction for the incomplete incorporation of this enzyme the recovery of enzyme activity results as 13%. The recovery of the activity of DMN reduction of the formate dehydrogenase complex was 15%. The activity of electron transport from formate to fumarate was as great as that of DMN reduction in the proteoliposomes containing formate dehydrogenase complex. This activity was absent from those containing formate dehydrogenase without cytochrome *b*, even though the activities of the individual enzymes were similar in both preparations. It should be mentioned that the electron transport was expected to be limited by formate dehydrogenase, since the activity of fumarate reductase complex exceeded that of formate dehydrogenase by an order of magnitude. It is concluded that the cytochrome *b* present in the formate dehydrogenase complex is required for restoration of the electron-transport activity.

Reducibility of the cytochromes b

From the finding that the low-potential cytochrome *b* of the formate dehydrogenase complex is required for restoring electron-transport activity, it is expected that this cytochrome interacts on the formate side of menaquinone in the reconstituted electron-transport chain. Therefore, the cytochrome should be reducible by formate in proteoliposomes containing the formate dehydrogenase and fumarate reductase complexes, in the absence of quinone. In the experiment of Table III, proteoliposomes were used that contained formate dehydrogenase complex and fumarate reductase complex at a molar ratio of about 1 : 10. One of the preparations contained vitamin K-1 and the other did not.

The total amounts of cytochrome *b* were reduced upon the addition of dithionite (Table III). Each of the two preparations contained three different types of cytochrome *b* which could be differentiated by their redox response to the various additions. The cytochrome *b* of the formate dehydrogenase complex is characterized by its proportion (about 10%) and by its reduction by formate in the presence of fumarate and NQNO [3]. Accordingly, nearly 10% of the total cytochrome *b* was reduced under these conditions with the preparation containing vitamin K-1. The same amount was reduced

TABLE III

REDUCIBILITY OF CYTOCHROME *b* OF PROTEOLIPOSOMES CONTAINING OR LACKING VITAMIN K-1

Reduction of cytochrome *b* was measured as the absorbance increase at 565–575 nm [1]. The amounts of reduced cytochrome *b* were recorded 5–10 s after the addition of either formate (10 mM) or succinate (20 mM) to the proteoliposomal suspension in the fully oxidized state. The proteoliposomes were suspended in an anaerobic buffer containing 50 mM phosphate at pH 7.4 and 22°C. When indicated 0.1 mM fumarate or 5 μmol/g protein NQNO was present. The proteoliposomes were prepared using the dialysis method and freeze-thawing. Fumarate reductase complex and formate dehydrogenase complex were used for preparation at a protein ratio of 10:1. Enzymic activities (in U/mg protein): formate → DMN, 2 (both preparations); $DMNH_2$ → fumarate, 32 (both preparations); formate → fumarate, 2 (vitamin K-1 present) and less than 0.1 (vitamin K-1 absent).

Additions	Reduced cytochrome *b* (μmol/g protein)	
	Vitamin K-1 absent	Vitamin k-1 present
Dithionite	11.1	10.4
Formate + fumarate + NQNO	–	0.92
Formate + fumarate	0.91	–
Succinate	4.6	4.7
Formate	–	4.8
Formate + succinate	–	5.3

by formate in the preparation lacking vitamin K-1 in the absence of NQNO. It is likely that the species of cytochrome *b* that is reduced under the latter conditions is identical with the low-potential cytochrome *b* of the formate dehydrogenase complex, since the proportion of cytochrome *b* reduced increases with the proportion of formate dehydrogenase complex incorporated (not shown). Therefore, it is concluded that this cytochrome interacts in the electron transport as a redox component on the formate side of menaquinone.

The high-potential cytochrome *b* of the fumarate reductase complex can be identified by its reducibility with succinate (Table III). This cytochrome amounts to about 45% of the total cytochrome *b* of the preparations and is reduced by formate in the proteoliposomes containing vitamin K-1 together with the cytochrome *b* of the formate dehydrogenase complex. The residual part of the cytochrome *b* (low-potential species of the fumarate reductase complex) cannot be reduced by succinate, formate or both at a velocity commensurable with that of electron transport in the proteoliposomes containing of lacking vitamin K-1. This suggests that it is not involved in the electron transport from formate to fumarate.

Discussion

The results presented here show that the formate dehydrogenase complex which is active in restoring electron transport in liposomes contains a low-potential cytochrome *b* ($E_m = -224$ mV). This cytochrome appears to consist of an M_r 25 000 peptide carrying one heme group (Table I). The redox response in the absence of vitamin K-1 and in the presence of NQNO points to its interaction on the formate side of menaquinone (Table III). Furthermore, the amount of NQNO required for full inhibition of the reconstituted electron-transport system was found to be equivalent to that of the cytochrome [6]. The formate dehydrogenase lacking cytochrome *b* catalyzes the reduction of DMN by formate and can also be incorporated into liposomes. However, this enzyme was inactive in restoring electron-transport activity. It is concluded that the low-potential cytochrome *b* of formate dehydrogenase complex is an obligatory component of the electron transport from formate to menaquinone in agreement with the scheme in the Introduction. The menaquinone-reactive site is apparently deficient in the enzyme lacking cytochrome *b*. The water-soluble menaquinone analogue DMN reacts at either another or an additional site. In agreement, it was earlier found that the activity of DMN reduction by formate was insensitive to NQNO in contrast to electron transport. [3]. It is likely that the cytochrome *b* reacts directly with menaquinone. However, it cannot be excluded that the menaquinone-reactive site might be localized on the M_r 110 000 or 20 000 peptide.

The high-potential ($E_m = -10$ mV) and the low-potential cytochrome *b* ($E_m = -180$ mV) of the fumarate reductase complex gave a common protein band at M_r 25 000 in gel electrophoresis with dodecyl sulfate [4]. Various treatments of the enzyme complex led to the separation of both cytochromes from the enzyme [4,5]. Therefore, it may be inferred that the electron-transport chain

contains two different species of low-potential cytochrome *b*, one interacting on the formate side and the other on the fumarate side of menaquinone. Although this possibility cannot safely be excluded, it is more likely that the low-potential cytochrome *b* associated with the fumarate reductase complex represents an inactive contaminant of the preparation. This cytochrome was not reducible by formate with or without NQNO in liposomes containing formate dehydrogenase complex and vitamin K-1 (Table III). In contrast, in the bacterial membrane, all the low-potential cytochrome *b* reacted in the same way as the cytochrome *b* of the formate dehydrogenase complex in the liposomal system. It was fully reduced by formate either in the presence of NQNO or in the absence of menaquinone [1].

Restoration of the electron transport in liposomes would be expected to occur also with formate dehydrogenase lacking cytochrome *b*, because the enzymes can be assumed to be laterally mobile within the membrane [9,10]. Collision of formate dehydrogenase lacking cytochrome *b* with the fumarate reductase complex should lead to a transfer of the low-potential cytochrome *b* and consequently to the restoration of electron transport. The finding that the electron transport is not restored under these conditions may mean that the cytochrome is inactive with respect to the reaction with formate dehydrogenase. In summary, the results presented further support the sequence of the electron-transport components as given in the scheme in the Introduction, but do not explain why most of the low-potential cytochrome *b* is isolated together with the fumarate reductase complex.

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