Biochimica et Biophysica Acta, 682 (1982) 258–263
Elsevier Biomedical Press

BBA 41176

RECONSTITUTION IN LIPOSOMES OF THE ELECTRON-TRANSPORT CHAIN CATALYZING FUMARATE REDUCTION BY FORMATE

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(Received June 11th, 1982)

Key words: Fumarate reduction; Electron-transport chain; Membrane reconstitution; Liposome; (Vibrio succinogenes)

Fumarate reduction by formate in *Vibrio succinogenes* is catalyzed by a membrane-bound electron-transport chain, and is coupled with the phosphorylation of ADP. The electron-transport chain was reconstituted in liposomes from the isolated components. The formate dehydrogenase complex (three different peptides), the fumarate reductase complex (three different peptides) and vitamin K-1 were required for the electron transport. The pathway of the electrons from formate to fumarate in the reconstituted chain was identical with that in the bacterial membrane. Each of the active enzyme complexes in the liposomes participated in the electron transport. This was valid for proteoliposomes with ratios of the contents of the two enzyme complexes ranging between 0.1 and 10. This indicates that vitamin K-1 forms a diffusible pool within the liposomal membrane that allows every quinone molecule to react with each molecule of the two enzyme complexes.

Introduction

Vibrio succinogenes performs electron-transport phosphorylation with fumarate as the terminal hydrogen acceptor and formate as the donor (reaction a) [1,2]:

$$\text{formate} + \text{fumarate} \rightarrow CO_2 + \text{succinate} \qquad \text{(a)}$$

It was proposed that the electron-transport chain catalyzing reaction a consists of the formate dehydrogenase complex, the fumarate reductase complex and menaquinone [2–7]. The membrane-bound enzyme complexes have been isolated and found to react with the water soluble menaquinone analogue DMN [3,5–7]. In contrast, formate dehydrogenase or fumarate reductase which are subunits of the complexes did not react with quinones.

In this communication, the isolated complexes together with vitamin K-1 were incorporated into liposomes according to the procedure developed by Helenius et al. [8] and Mimms et al. [9]. This led to restoration of electron transport from formate to fumarate.

Methods

Preparation of the enzyme complexes

The fumarate reductase complex [6], and the formate dehydrogenase complex [5] which is characterized by its reaction with DMN were isolated as previously described. However, the dithionite which was present in the buffers used earlier was replaced by 1 mM dithiothreitol. The Triton associated with these preparations was replaced by octylglucoside using the following procedure. Each preparation (3 ml containing 15–20 mg protein) was layered onto 37 ml of an anaerobic buffer

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

0005-2728/82/0000–0000/$02.75

(0°C) in a centrifuge tube. The buffer contained 30 mM octylglucoside, 20 mM Tris, 1 mM NaN_3, 1 mM malonate, 1 mM dithiothreitol and a linear sucrose gradient (10–25%, w/v), pH 7.7. The tubes were centrifuged for 3 h at 206 000 × *g* in a Beckman VTi 50 rotor. The fractions containing the enzymes were pooled, concentrated by pressure dialysis using a 'Diaflo Ultrafilter PM10' (Amicon, Lexington, MA, U.S.A.) and stored in liquid N_2.

Preparation of the proteoliposomes [8,9]

N_2 was blown on the surface of a solution (1 ml) containing 1 mg phosphatidylcholine (from soybean, Sigma No. 3644) and 10 nmol vitamin K-1 in a mixture of chloroform and methanol (2 : 1, v/v) at room temperature, until the solvents were evaporated. The resulting lipid film was dissolved in an anaerobic buffer (0°C) containing the enzyme complexes (total 0.2 mg protein), 8 mg octylglucoside, 20 mM Tris, 1 mM NaN_3, 1 mM malonate and 1 mM dithiothreitol, pH 7.7. The solution was dialyzed for 35–40 h against two 1-l volumes of an anaerobic buffer (0°C) containing 10 mM Tris, 1 mM NaN_3, 1 mM malonate and 0.7 mM dithiothreitol, pH 7.7. The preparation was frozen in liquid N_2 for 10 min and then thawed. This was repeated twice, before the enzymic activities were measured.

Enzymic activities

The activities of fumarate reduction by formate [3] or $DMNH_2$ [6] and of formate oxidation by DMN or benzyl viologen [5] were measured at 37°C as described. The unit of enzymic activity (U) represents the oxidation of 1 μmol formate or the reduction of 1 μmol fumarate per min.

Protein

Protein was measured by counting the radioactivity which had been incorporated by growing the bacteria in the presence of [^{3}H]leucine [5,6]. The specific radioactivity of the protein was determined using the biuret method with KCN [5].

Results

Composition of the proteoliposomes

Using sucrose density gradient centrifugation in the presence of octylglucoside, the Triton X-100 associated with the formate dehydrogenase and the fumarate reductase complexes was replaced by octylglucoside. Subsequent dialysis of the enzymes in the presence of phospholipid and octylglucoside (protein:phosphatidylcholine:octylglucoside = 1 : 5 : 40, w/w) and freezing-thawing led to the formation of proteoliposomes. The average diameter of the liposomes was determined by electron microscopy as 0.2 μm. Both enzyme complexes were fully incorporated into the liposomes. This was concluded from gel filtration using Sepharose CL-4B (not shown). The protein eluted together with the liposomes at the void volume of the column. The fractions corresponding to the Stokes' radii of the free enzyme complexes did not contain protein. On isopycnic centrifugation of the preparation on a sucrose density gradient (10–60%, w/v), the bands of the enzymes coincided with that of the phospholipid at a density of 1.06 g/ml. This density is characteristic of proteoliposomes with a content of 0.2 g protein/g phosphatidylcholine [8]. Protein-phospholipid complexes with greater densities [8] were not obtained with

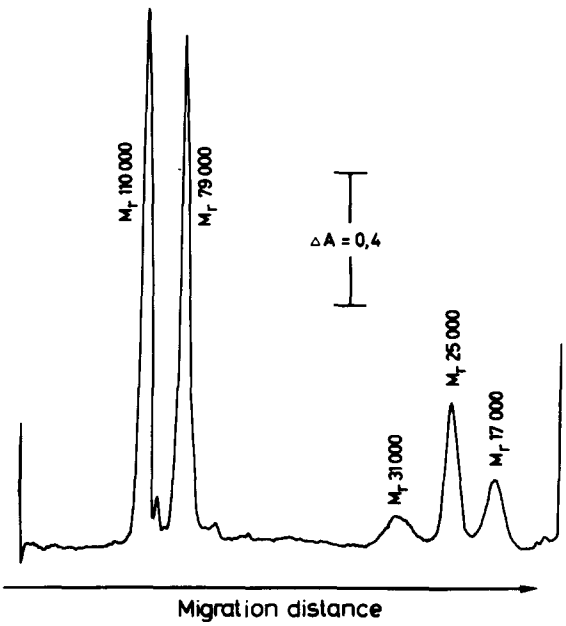


Fig. 1. Dodecyl sulfate polyacrylamide gel electrophoresis of the protein of the proteoliposomes. Proteoliposomes (30 μg protein) containing the formate dehydrogenase and the fumarate reductase complexes at a molar ratio of 0.9 were subjected to gel electrophoresis as described previously [5]. The gels were scanned at 546 nm after staining with Coomassie blue G-250.

the present method. However, before freezing-thawing of the preparation, 80–90% of the protein was present as protein-phospholipid complex with a density of 1.20 g/ml and a content of 0.5 g protein/g phospholipid. The content of total protein in the proteoliposomes was about 10-times smaller than that in the bacterial membrane. The contents of the two enzyme complexes could be varied by the preparation procedure and ranged between 10 and 300% of the amounts in the bacterial membrane. From the average diameter it was calculated that every liposome contained 150 molecules of each complex, when equal amounts of the enzymes were used for the preparation at a total content of 0.2 g protein/g phospholipid.

Dodecyl sulfate gel electrophoresis of the protein of the liposomes (Fig. 1) resolved five different peptide bands (M_r 110000, 79000, 31000, 25000 and 17000), the functions of four of which are known. The M_r 110000 peptide represents formate dehydrogenase [5]. In addition to this peptide the formate dehydrogenase complex contained cytochrome *b* (−200 mV) (M_r 25000) and the M_r 17000 peptide. The molar ratio of these peptides in the formate dehydrogenase complex was 1 : 1 : 1.4 as estimated from the stain of the corresponding bands of the gels. The function of the M_r 17000 peptide is not known and it cannot yet be decided whether or not it is required for reconstitution. The peptide could not be separated from the complex by subjecting the preparation to successive chromatography on Sephacryl S300, Phenylsepharose CL-4B and DEAE-Sepharose CL-6B.

The fumarate reductase complex consists of fumarate reductase (M_r 79000), the M_r 31000 peptide and cytochrome *b* [6]. The molar ratio of these peptides was 1 : 1.2 : 1.8 [6]. The M_r 31000 peptide carries the binuclear iron-sulphur centre [10] and mediates the electron transport between fumarate reductase and cytochrome *b* (−20 mV) [7].

The M_r 25000 band represents the two cytochromes *b*. The peptides of cytochrome *b* (−20 mV) and of cytochrome *b* (−200 mV) give the same band in gel electrophoresis with dodecyl sulfate [6,7]. As judged from the stain of the M_r 110000 and M_r 79000 bands, formate dehydrogenase and fumarate reductase were present in nearly equimolar amounts, as would be expected from the fact that equimolar amounts of the two enzyme complexes were used for the preparation of the proteoliposomes.

TABLE I

TURNOVER NUMBERS OF THE FUMARATE REDUCTASE AND THE FORMATE DEHYDROGENASE COMPLEXES CALCULATED FROM THE SPEFICIC ACTVITIES AND THE MOLECULAR WEIGHTS OF THE ENZYMES [5,6]

Enzymic reaction			Turnover number (min^{-1}) ($\times 10^3$)	
			Triton X-100	Liposomes
$DMNH_2$	→	fumarate	10	12
Formate	→	DMN	23	12
Formate	→	benzyl viologen	26	25

The proteoliposomes catalyzed the reactions which are characteristic of the formate dehydrogenase complex (formate → DMN) and the fumarate reductase complex ($DMNH_2$ → fumarate). These activities were independent of the presence of vitamin K-1 in the liposomes. The turnover numbers of the two enzymes in the liposomes are compared to those in Triton (Table I), where both enzymes are regarded as fully active. The activities of fumarate reduction by $DMNH_2$ and that of benzyl viologen reduction by formate were fully conserved in the liposomes, while 52% of the activity of DMN reduction by formate was recovered. This suggests that about half of the formate dehydrogenase complex molecules lost the capacity to react with naphthoquinones during the incorporation procedure.

Properties of the electron transport

Fig. 2 demonstrates that the liposomes containing the formate dehydrogenase and fumarate reductase complexes catalyze electron-transport activity from formate to fumarate provided that vitamin K-1 is present. The activity was almost completely dependent on freezing-thawing of the preparation, and it increased with the amount of vitamin K-1 until saturation was reached with nearly 10 mM vitamin K-1 in the phospholipid.

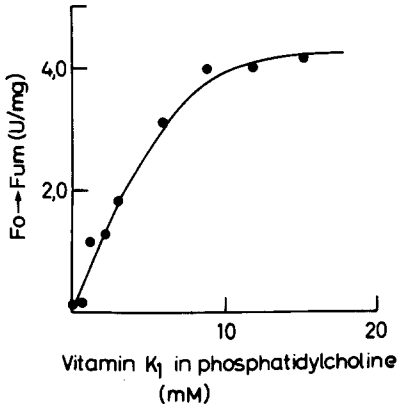


Fig. 2. Electron-transport activity of proteoliposomes as a function of the vitamin K-1 content. Proteoliposomes containing constant amounts of the formate dehydrogenase and the fumarate reductase complexes and increasing amounts of vitamin K-1 were prepared as described in Methods. All the preparations catalyzed $DMNH_2$ oxidation by fumarate at a specific activity of 30 U/mg total protein. The activity of DMN-reduction by formate varied between 5 and 7 U/mg total protein. Fo, formate; Fum, fumarate.

This concentration is similar to that of menaquinone in the cytoplasmic membrane of *V. succinogenes*. Vitamin K-1 differs from the menaquinone of the bacteria with respect to the length

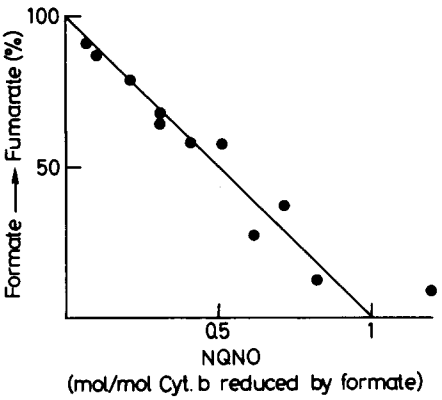


Fig. 3. NQNO titration of the activity of fumarate reduction by formate of proteoliposomes. The titration was done by adding increasing amounts of NQNO in 10 mM KOH to the complete reaction mixtures (see Methods). The amount of cytochrome *b* reduced by formate (1.3 μmol/g protein) was measured as the absorbance increase at 565–575 nm (4) caused by the addition of formate in the presence of NQNO (2 μmol/g protein) and fumarate. The specific activity (100%) of fumarate reduction by formate was 9.1 U/mg protein.

and the number of double bonds of the isoprenoid chain of the quinones. It is possible that menaquinone is more efficient than vitamin K-1 in restoring electron transport.

The electron-transport activity of the proteoliposomes is efficiently inhibited by NQNO (Fig. 3). The titration curve was linear up to 90% inhibition. The amount of NQNO required for full inhibition, determined by extrapolation, was equivalent to that of the cytochrome *b* which is reduced by formate in the presence of NQNO or in the absence of vitamin K-1. This cytochrome is associated with the formate dehydrogenase complex and is known to exhibit a midpoint potential of −200 mV and to react with NQNO [5]. It is concluded that cytochrome *b* (−200 mV) is an obligatory component of the reconstituted electron-transport chain and interacts on the formate side of the quinone.

Electron transport as a function of the enzyme contents of the liposomes

The electron-transport activity of the proteoliposomes was dependent on the presence of both enzyme complexes (Fig. 4). No activity was measured in the absence of either the formate dehydrogenase or the fumarate reductase complex. In order to determine the proportion of active enzyme complexes that participate in the electron transport, various liposome preparations were prepared which differed in the contents of the two enzyme complexes relative to each other. The ratio of these contents varied between 0.1 and 10 mol fumarate reductase complex/mol formate dehydrogenase complex. The amount of total protein (0.2 g) per g phospholipid was the same in all the preparations. The activity of electron transport from formate to fumarate (v), that of DMN reduction by formate (V_{FDH}) and that of $DMNH_2$ oxidation by fumarate (V_{FR}) was measured with each preparation. The values of v divided by the corresponding values of V_{FDH} or V_{FR} were plotted against the ratios of V_{FDH} and V_{FR} (dots in Fig. 4). These experimental points were compared to theoretical curves which were drawn in Fig. 4 according to Eqn. 1:

$$v = V_{FDH} \cdot V_{FR}/(V_{FDH} + V_{FR}) \quad (1)$$

Eqn. 1 refers to two enzyme systems with a diffusi-

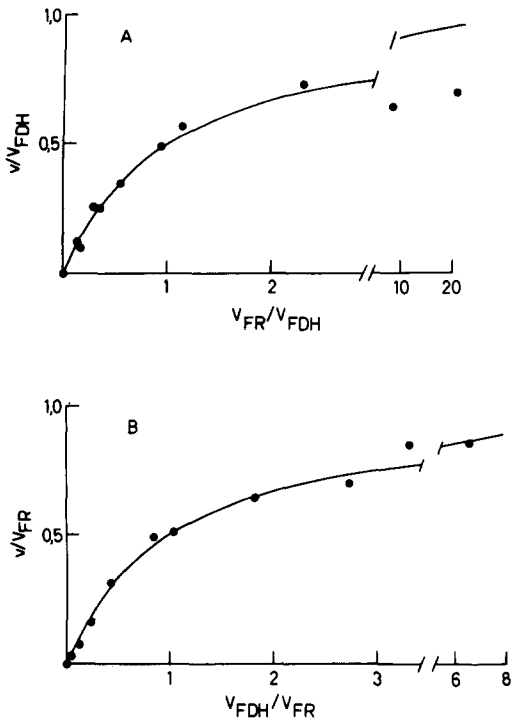


Fig. 4. Electron-transport activity as a function of the relative contents of the formate dehydrogenase and the fumarate reductase in liposomes. Proteoliposomes containing various amounts of the formate dehydrogenase and the fumarate reductase complexes were prepared as described in Methods. Electron transport (v), V_{FDH} and V_{FR} were measured just after freezing-thawing of the preparation. At a ratio $V_{FDH}/V_{FR} = 1$ the turnover numbers were $5.4 \cdot 10^3$ and $10.7 \cdot 10^3$ min^{-1} for V_{FDH} and V_{FR}, respectively. The curves were calculated from V_{FDH} and V_{FR} according to Eqn. 1.

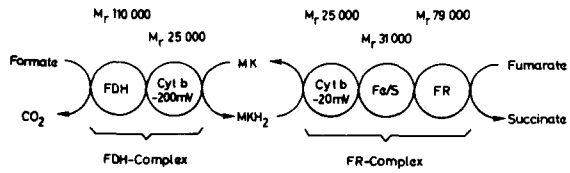
ble intermediate and is valid for the mitochondrial electron transport with respect to the enzymes reducing and oxidizing ubiquinone [11,12]. It can be seen that the experimental points closely follow the theoretical saturation curves, supporting the conclusion that the two enzyme complexes in the liposomes react through a diffusible intermediate. From this it is concluded that V_{FDH} and V_{FR} are quantitative measures of the velocities of the reactions of the two enzyme complexes with vitamin K-1 (or menaquinone). Furthermore, all the enzyme complexes that react with DMN or $DMNH_2$ appear to participate in the electron transport from formate to fumarate in the liposomes.

Discussion

The components of the electron-transport chain

Incorporation of the formate dehydrogenase complex and the fumarate reductase complex into liposomes led to the restoration of the activity of electron transport from formate to fumarate. Each active enzyme complex was shown to participate in the electron transport. The pathway of the electrons (Scheme I) was identical with that in the bacterial membrane, because a lipophilic naphthoquinone was required and the activity was equally sensitive to NQNO. The cytochrome *b* (−200 mV) of the formate dehydrogenase complex is clearly an obligatory component of the reconstituted electron-transport chain, since it is known to be the target of NQNO inhibition [4,5]. The function of the M_r 17000 peptide which is associated with the formate dehydrogenase complex is not known. It remains to be elucidated whether or not this peptide is necessary for the reduction of DMN by formate and for reconstitution. In a previous paper [5], the formate dehydrogenase as measured with benzyl viologen as acceptor was isolated with a slightly different procedure. This preparation contained smaller and variable amounts of peptides of similar molecular weights which were regarded as impurities. The activity of formate dehydrogenase with DMN as acceptor was considerably smaller, and some preparations did not react at all with DMN. Therefore, it cannot be decided whether cytochrome *b* (−200 mV) is the electron donor to menaquinone, and the pathway of the electrons from formate dehydrogenase to menaquinone remains to be clarified.

Earlier studies with the isolated fumarate re-



Scheme I. Sequence of the components of the electron-transport chain catalyzing fumarate reduction by formate in *V. succinogenes*. FDH, formate dehydrogenase; Fe/S, binuclear iron-sulfur centre; FR, fumarate reductase; MK, menaquinone; MKH_2, reduced menaquinone. The cytochromes *b* are differentiated by their midpoint redox potentials.

ductase complex revealed the functions of the subunits and showed that cytochrome *b* (−20 mV) is responsible for the reaction with water-soluble quinones and hydroquinones [6,7]. The restoration of electron transport in the liposomes suggests that this cytochrome is the acceptor of the electrons also from the lipophilic hydroquinone in the membrane. The cytochrome *b* (−200 mV) which is associated with the isolated fumarate reductase complex was not involved in the fumarate reduction by $DMNH_2$ and was regarded as a contaminant of the preparation [6,7]. After incorporation of the fumarate reductase complex into liposomes together with formate dehydrogenase complex, this cytochrome was not reduced by formate in the presence of NQNO or in the absence of vitamin K-1 (not shown). From this it is concluded that this cytochrome does not participate in the electron transport of the proteoliposomes.

Menaquinone as a diffusible pool within the membrane

Eqn. 1 was derived from studies of the mitochondrial electron transport in which the enzymes reducing and oxidizing ubiquinone were titrated by specific inhibitors [11]. It is not surprising that the equation is valid also in the liposomal system, where the titration is done by varying the contents of the enzymes. With the mitochondrial system it was shown that the enzymic activities designated by *V* reflect the maximum rates of the redox reactions of ubiquinone. The activity of oxidation of a water-soluble hydroquinone at saturating concentrations was equivalent to the maximum rate of oxidation of the membranous ubiquinone [11]. Similarly, it is likely that the activities of DMN reduction by formate (V_{FDH}) and of $DMNH_2$ oxidation by fumarate (V_{FR}) are equivalent to the maximum activities of the two enzyme complexes with the liposomal quinone. On this basis, the validity of Eqn. 1 for the liposomal system indicates that vitamin K-1 forms a diffusible pool within the membrane that allows every quinone molecule to react with each molecule of the enzyme complexes [11,12]. This pool concept explains why each enzyme complex participates in electron transport in spite of the variation of their relative contents by two orders of magnitude.

Quinone (vitamin K-1 or menaquinone) appears to be the substrate of the formate dehydrogenase complex, and hydroquinone appears to react with the fumarate reductase complex. In both reactions the quinone radicals are expected to participate as products, but not as substrates of the complexes. This is supported by the validity of Eqn. 1 for the liposomal system. Eqn. 1 implies that the reduction of the quinone and the oxidation of hydroquinone within the membrane are pseudo-first-order reactions with respect to quinone and hydroquinone. This has been shown to be true for ubiquinone in mitochondrial electron transport [11], and is likely to hold also for vitamin K-1 and the bacterial menaquinone. First-order reactions are also suggested by the observations that DMN reduction by formate [5] and $DMNH_2$ oxidation by fumarate [6] follow Michaelis kinetics.

Acknowledgements

This work was supported by grants from Deutsche Forschungsgemeinschaft and from Fonds der Chemischen Industrie to A.K.

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