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STRUCTURAL PROPERTIES OF THE PROTEOLIPOSOMES CATALYZING ELECTRON TRANSPORT FROM FORMATE TO FUMARATE *

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The electron-transport chain catalyzing fumarate reduction by formate has recently been reconstituted from the formate dehydrogenase complex and the fumarate reductase complex from *Vibro succinogenes*, in a liposomal preparation containing vitamin K-1 (Unden, G. and Kröger, A. (1982) Biochim. Biophys. Acta 682, 258–263). We have now investigated the structural properties of this preparation. The preparation was found to consist of a homogeneous population of unilamellar proteoliposomes with an average diameter of about 100 nm and an internal volume of 2–4 ml/g phospholipid. The buoyant density (1.07 g/ml) was consistent with the protein/phospholipid ratio (0.2 g/g) of the preparation. Leakage of glucose from the internal spaces of the proteoliposomes was negligibly slow. Proteoliposomes prepared with either of the enzyme complexes showed peripheral projections mainly on the outer surface, when examined by electron microscopy after negative staining. The size, orientation and surface density of the projections were consistent with those of the enzymes. Most of the substrate and dye-reactive sites (70–90%) of the enzymes in the proteoliposomes were accessible to external non-permeant substrates. The proteoliposomes catalyzing electron transport were formed by freeze-thawing a mixture of liposomes and protein-phospholipid complexes which did not perform electron transport from formate to fumarate. Nearly the entire amount of the enzymes supplied (0.2 g protein/g phospholipid) was incorporated into the liposomes by this procedure. The transformation of liposomes into proteoliposomes was accompanied by exchange of the internal solutes with the external medium.

Introduction

Fumarate reduction by formate in *Vibrio succinogenes* represents a relatively simple system of electron-transport phosphorylation that appears to be especially well suited for elucidating the mechanism of energy transduction [1–6]. The electron-transport chain of this system was recently reconstituted from the isolated components in a liposomal preparation by means of the dialysis technique [1]. While proteoliposomes are known to be formed directly by this procedure with other proteins [7–9], additional freeze-thawing of the preparation was required for restoring formate-fumarate reduction. It was, therefore, of interest to understand the effect of freeze-thawing on the preparation. For this purpose we have investigated the structural properties of the liposomal preparation. These studies represent a basic prerequisite for an investigation of the mechanism of energy transduction in this system.

* This paper is dedicated to Peter Karlson on the occasion of his 65th birthday.

Methods

Preparation of the proteoliposomes. Proteoliposomes containing formate dehydrogenase complex and/or fumarate reductase complex were prepared by dialysis and subsequent freeze-thawing of a mixture containing octylglucoside, phosphatidylcholine (from soybean, Sigma No. 3644), vitamin K-1 and the enzyme complexes as described earlier [1]. The formate dehydrogenase complex [1,3,14] and the fumarate reductase complex [1,4] were isolated as described previously.

Gel filtration of the proteoliposomes. Gel filtration of the proteoliposomes was done on a Sepharose CL-4B column (27 ml, 1 cm inner diameter) which was equilibrated with an anaerobic buffer containing 10 mM Tris, 2 mM fumarate, 1 mM NaN_3 and 0.5 mM dithiothreitol, pH 7.7, 0°C.

Protein. Protein was measured by counting the radioactivity which had been incorporated by bacteria growing in the presence of [^{3}H]leucine [3,4]. The specific radioactivity of the protein was determined using the biuret method with KCN [10].

Glucose. Glucose was measured using the photometric assay with hexokinase and glucose-6-phosphate dehydrogenase [11]. The glucose content of the proteoliposomes was measured after lysis with 0.02% Triton X-100.

Enzyme activities. Formate dehydrogenase was measured photometrically either with methylene blue or benzyl viologen as acceptor [3]. Fumarate reductase was assayed photometrically by following methylene blue reduction or ferricyanide reduction by succinate [5,6]. One unit of activity (U) represents the oxidation of 1 μmol formate or succinate/min at 37°C.

Electron microscopy. The negative staining procedure was performed according to the method of Valentine et al. [12]. The samples were applied to carbon film-coated (3–6 nm) sheets of cleaved mica. Sodium phosphotungstate solution (2%, w/v, pH 7.0) was used for negative staining. The preparations were immediately examined in a Philips 301 G electron microscope operating at 80 kV.

Results

Hydrodynamic properties

Dialysis of a mixture of octylglucoside, phosphatidylcholine, vitamin K-1, formate dehydrogenase complex and fumarate reductase complex was earlier reported to yield a preparation which catalyzed electron transport after freeze-thawing [1]. The homogeneity of this preparation before and after freeze-thawing has now been investigated using gel filtration on Sepharose CL-4B (Fig. 1). The dialysis was performed as done previously, except that 30 mM glucose was included in the mixture. Half of the resulting suspension was immediately subjected to gel filtration (Fig. 1A), while the other was freeze-thawed before (Fig. 1B).

Approx. 70% of the phospholipid of the preparation after dialysis (Fig. 1A) and 90% of that of the freeze-thawed preparation (Fig. 1B) eluted with the void volume of the column. In both experiments, these void volume fractions were the only ones that contained glucose, as measured by enzymatic assay after addition of Triton. From the glucose content per g phospholipid in these fractions and the concentration of glucose of the preparation before gel filtration, an internal volume of about 3 ml/g phospholipid was calculated. This experiment demonstrates that vesicles with similar internal volume are formed by dialysis with or without freeze-thawing. The finding that glucose was virtually absent from the fractions corresponding to K_{av} values between 0.2 and 0.8 indicates that the leakage of glucose from the vesicles was slow in both preparations.

The effect of freeze-thawing on the preparation is seen from the elution profiles of protein and of the enzyme activities. Approx. 90% of both total protein and enzyme activities eluted with the vesicles of the preparation that underwent freeze-thawing (Fig. 1B). This indicates that the enzyme complexes were incorporated into the vesicles. The protein/phospholipid ratio of these vesicles was nearly the same as that used for preparation (0.2 g/g). In contrast, more than 80% of the enzyme complexes eluted as K_{av} values between 0.2 and 1 with the preparation obtained by dialysis alone (Fig. 1A). The enzymes did not elute as discrete bands at the K_{av} values corresponding to their Stokes radii. This indicates that

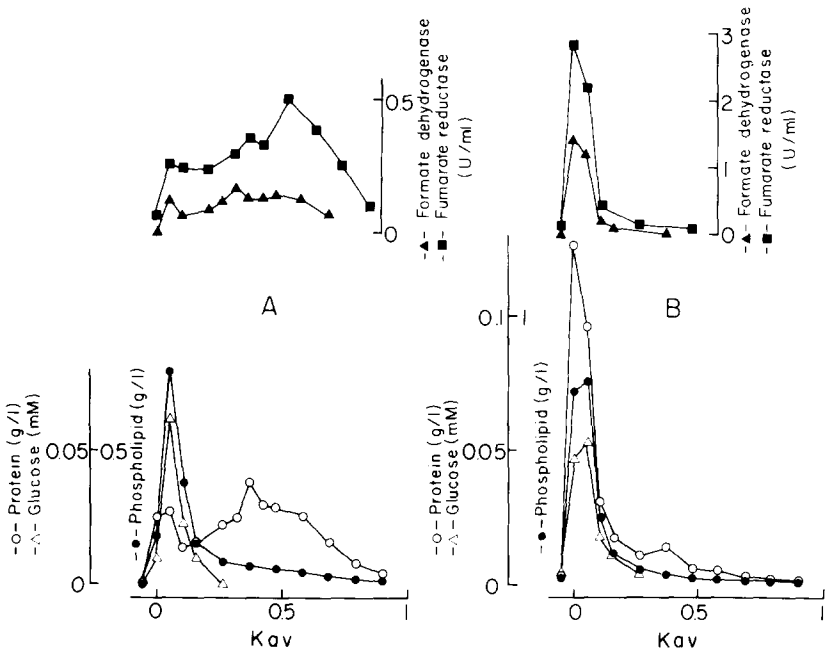


Fig. 1. Gel filtration of liposomes. The liposomes were prepared as described in Methods. However, [^{14}C]dipalmitoylphosphatidylcholine was also present before dialysis, and glucose (30 mM) was added to the dialysis buffer. Half of the preparation (0.35 ml, corresponding to 0.38 mg total protein) was immediately subjected to gel filtration (A), the other half was freeze-thawed seven times, before gel filtration (B). Formate dehydrogenase was assayed with methylene blue as acceptor, and fumarate reductase by its activity of succinate-methylene blue reduction. The K_{av} values were calculated from the elution volumes (V_e), the total volume (V_t = 20 ml) and the void volume (V_0 = 7 ml) according to the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$. The specific radioactivity of the phospholipids (14.4 dpm/μg) was determined by phosphate analysis [13] using an average molecular weight of 740. The specific radioactivity of the protein was 48 dpm/μg.

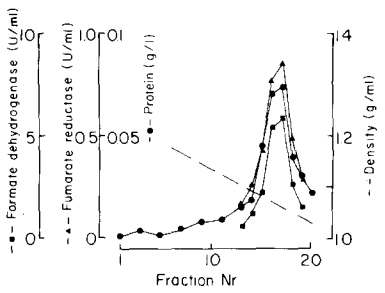


Fig. 2. Equilibrium centrifugation of proteoliposomes on a sucrose density gradient. A suspension of proteoliposomes prepared with freeze-thawing (0.25 ml, containing 0.12 mg protein) was layered onto 4.7 ml of a linear sucrose gradient (15–60%, w/v) in an anaerobic buffer containing 10 mM Tris, 1 mM NaN_3 and 1 mM dithiothreitol, pH 7.7, 0°C. Centrifugation for 3.5 h at 65000 rpm was done in a VTi 65 rotor (Beckman). Formate dehydrogenase was assayed with methylene blue as acceptor and fumarate reductase by its activity in succinate-dependent methylene blue reduction. The specific radioactivity of the protein was 48 dpm/μg.

protein-phospholipid complexes were formed. The protein/phospholipid ratio of these complexes was approx. 0.5 g/g.

The preparation after freeze-thawing was subjected to equilibrium centrifugation on a linear sucrose gradient (Fig. 2). More than 80% of the total protein was found in a symmetrical band with the peak at a density of 1.07 g/ml. This density corresponds to a protein/phospholipid ratio of 0.2 g/g which is identical with that of the vesicles measured after gel filtration (Fig. 1B). The protein band coincided with the activities of the two enzymes. When the preparation obtained by dialysis alone was centrifuged in the same way, most of the protein was found at densities greater than 1.1 g/ml (not shown). This density corresponds to protein-phospholipid complexes containing more than 0.4 g protein/g phospholipid, in agreement with the result obtained from gel filtra-

TABLE I

ENZYME ACTIVITY OF FUMARATE REDUCTASE AND FORMATE DEHYDROGENASE BEFORE AND AFTER LYSIS OF THE PROTEOLIPOSOMES

Lysis of the proteoliposomes with Triton X-100 (0.02%) or melittin (1.6 g/g protein) was achieved by adding these compounds directly to the cuvette. For lysis with phospholipase A_2, the proteoliposomes (0.5 g protein/l) were incubated with the enzyme (0.5 g protein/l) for 30 min at room temperature in the presence of 2 mM $CaCl_2$. Values are expressed as U/mg protein.

Preparation	Lytic agent	Succinate → $Fe(CN)_6^{3-}$		Formate → benzyl viologen	
		Lysed	Intact/lysed	Lysed	Intact/lysed
1	Triton X-100	60	0.73	28	0.79
2	Phospholipase A_2	56	0.75	33	0.80
	Phospholipase A_2 + Triton X-100	56	0.75	33	0.81
3	Melittin	65	0.76	–	–
	Melittin + Triton X-100	65	0.76	–	–

tion (Fig. 1A). From the experiments of Figs. 1 and 2 it is concluded that freeze-thawing after dialysis, but not dialysis alone, causes the incorporation of the enzyme complexes into phospholipid vesicles.

Internal volume

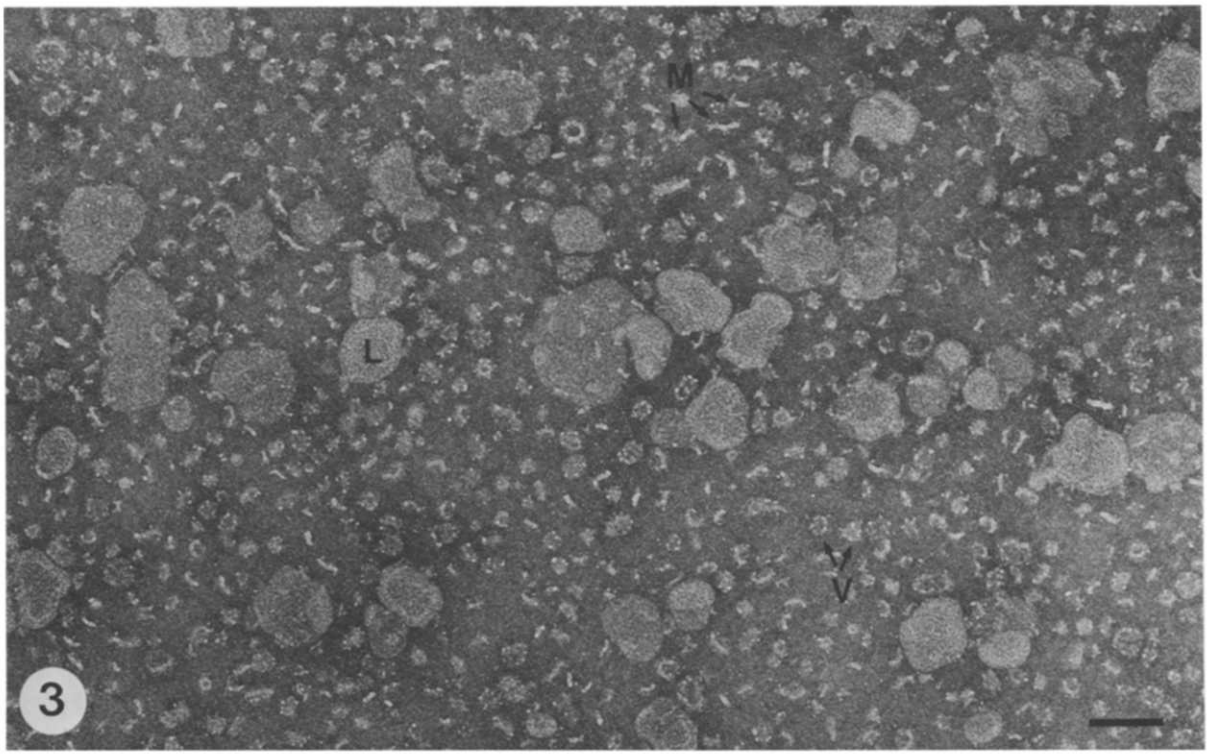
The internal volume of the vesicles was determined by measuring the contents of two small hydrophilic compounds that were added at different stages of the preparation procedure. The vesicles were prepared by dialysis with the enzyme complexes and vitamin K-1 [1] in the presence of glucose. Subsequently, labeled taurine was added and the suspension was subjected to freeze-thawing. The resulting proteoliposomes were separated from the external glucose and taurine by gel filtration, and the contents of glucose and taurine per g phospholipid were measured. From these contents the internal volumes of the vesicles were calculated, using the concentration of glucose or taurine in the suspension before gel filtration. The volumes measured in different fractions with glucose (2.2–3.9 ml/g phospholipid) differed by less than 20% from those determined with taurine (2.5–4.3 ml/g phospholipid). This result indicates that freeze-thawing causes equilibration between the internal space of the vesicles formed by dialysis and the external medium. The inner diameter of the vesicles can be estimated from the internal volume, assuming that the vesicles are spherical and consist of unilamellar bilayers of phospholipid, and that each phospholipid molecule occupies an area of 0.7 nm^2 on the surface of the vesicle [8]. Using the extreme values of the internal volume, the inner diameter was calculated to range between 50 and 100 nm.

Accessibilities of the enzymes

In an earlier publication [5], the orientation of the substrate and dye-reactive sites of formate dehydrogenase and fumarate reductase in cells and membrane vesicles of *V. succinogenes* was elucidated by assaying these enzymes before and after lysis. The orientation of the dye-reactive sites of the enzymes was found to be the same as that of

Fig. 3. Negatively stained preparation obtained with fumarate reductase complex after dialysis alone. The preparation contains liposomes (L), vesicular structures (V), and membrane sheets (M). Magnification, ×100000. Bar: 100 nm.

Fig. 4. Negatively stained proteoliposomes prepared with fumarate reductase complex by dialysis and freeze-thawing. (a) Overview of the preparation. Magnification, ×100000. Bar: 100 nm. (b and c) Enlarged proteoliposomes showing the enzyme complexes in face-on and side views. Magnification, ×200000. Bar: 100 nm.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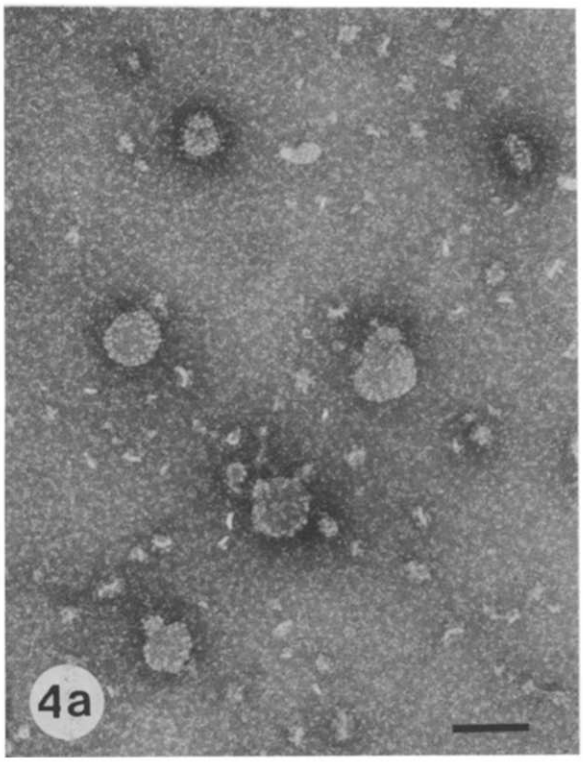
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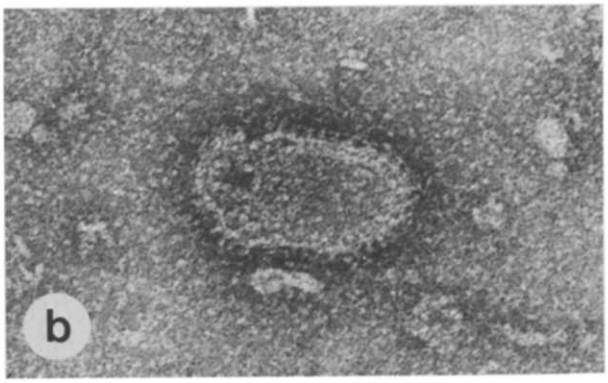
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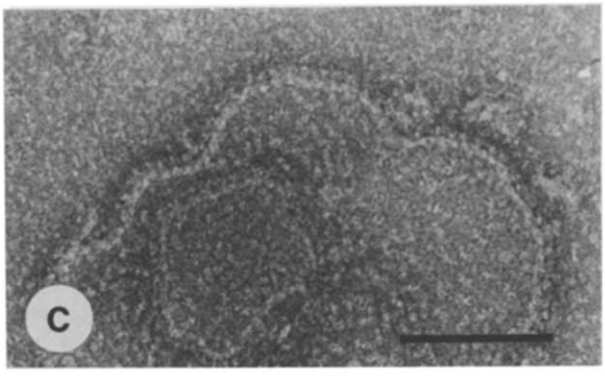
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the substrate sites. Using the same method, we have determined the orientation of the enzymes in the proteoliposomes (Table I). Formate dehydrogenase was assayed with benzyl viologen as acceptor and fumarate reductase by following ferricyanide reduction with succinate. The donors as well as the acceptors of both assay systems should not permeate across the lipid bilayer of the vesicles [5]. Therefore, only those enzyme molecules with the substrate and dye-reactive sites oriented towards the outside of intact vesicles are expected to be reactive.

The activity of succinate oxidation by ferricyanide increased by a factor of 1.4 and that of formate oxidation by benzyl viologen 1.3-fold, when Triton was added to the preparation. This suggests that 73% of the fumarate reductase and 79% of the formate dehydrogenase molecules are accessible to the external substrates. Similar results were obtained with other preparations, when phospholipase A_2 or melittin were applied instead of Triton. When Triton was added after treatment with phospholipase or addition of melittin, the activities were not further stimulated. This indicates that the stimulation caused by Triton is due to lysis of the vesicles and not to activation of the enzymes. Uncoupling agents did not bring about stimulation of the enzymic activities (not shown). This excludes the possibility of any interference from the effect of 'respiratory control' on the accessibility measurements.

The finding that identical effects are brought about by either of the three different lytic agents suggests that the stimulation is caused by rendering more enzyme molecules of the preparation accessible to their substrates. The ratio of the activities before and after lysis may be taken to reflect the proportion of enzyme molecules, the substrate and dye-reactive sites of which are oriented towards the outside of the vesicles. Using these methods with various proteoliposome preparations, it was found that 70–90% of the incorporated molecules of the fumarate reductase complex and 75–85% of those of the formate dehydrogenase complex face the outside of the vesicles.

Electron microscopy

Liposomal preparations containing fumarate reductase complex only were investigated by electron microscopy after negative staining with phosphotungstate. Figs. 3 and 4 show preparations after dialysis and dialysis plus freeze-thawing, respectively. Both preparations contain unilamellar vesicles with a diameter of about 100 nm. This value is close to the diameter (50–100 nm) calculated from the internal volumes of the preparations. The liposomes obtained by dialysis only show a smooth surface (Fig. 3). Subsequent freeze-thawing causes the formation of typical proteoliposomes with peripheral particles oriented mainly towards the outside (Fig. 4).

The preparation obtained by dialysis alone (Fig. 3) is heterogeneous. Beside liposomes it contains membrane sheets and vesicular structures which represent intermediate products of the process of proteoliposome formation. These structures probably represent the protein-phospholipid complexes that elute at K_{av} values greater than 0.2 during gel filtration (Fig. 1A) and show buoyant densities greater than 1.1 g/ml in equilibrium centrifugation. The membrane sheets and vesicular structures (Fig. 3) were not sealed and were studded with particles. These particles appear to be identical with those on the surface of the proteoliposomes (Fig. 4). They have a nearly rectangular shape in side view with dimensions of 4.8–5.5 nm (short axis) and 6.8–7.5 nm (long axis). Some particles show faint cleavage planes in the centre parallel to the long axis and small constrictions at the base. Assuming a cylindrical shape and using the average dimensions 5.1 nm (diameter) and 7.1 nm (height) and a partial specific volume of 0.73 ml/g for protein, the molecular weight can be calculated as 119000. This value is close to the molecular weight of a fumarate reductase that lacks cytochrome *b* (121000), which was measured earlier by other methods [4].

The appearance of the preparation after freeze-thawing (Fig. 4) is more homogeneous than after dialysis only (Fig. 3), in agreement with the results from gel filtration (Fig. 1) and equilibrium centrifugation (Fig. 2). The preparation consists mainly of proteoliposomes and only a few other membrane structures are visible. The particle density on the surface varies between 250 and 400 nm^2 per particle from one proteoliposome to another. This value was obtained by considering that the visible particles are derived from two superimposed proteolipid bilayers.

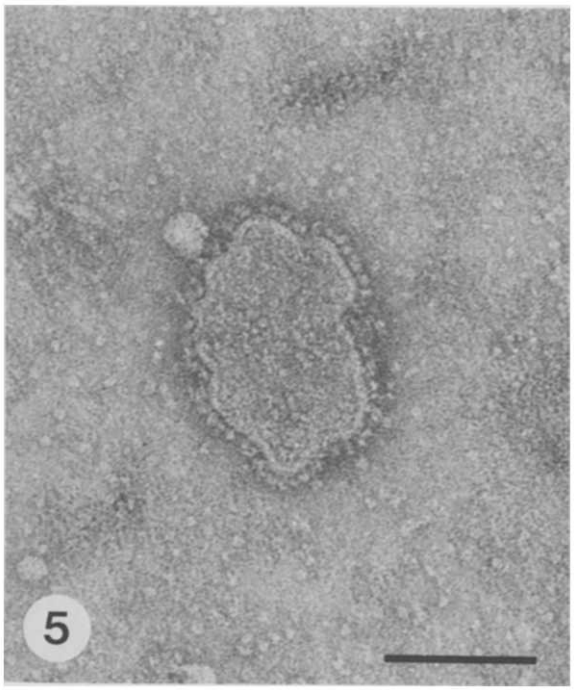

Fig. 5. Negatively stained proteoliposomes prepared with formate dehydrogenase complex by dialysis and freeze-thawing. Magnification, ×200000. Bar: 100 nm.

The surface density of the fumarate reductase complex, as calculated from the protein-phospholipid ratio of the proteoliposomes (0.2 g/g), is found to be in the same range as that of the particles. Using the molecular weights of the fumarate reductase complex (200000) and of phospholipid (1000), the molar ratio of these constituents is obtained as 1 : 1000. From this value and that of the area required by a single molecule of phosphatidylcholine in the bilayer (0.7 nm^2) [8], one may calculate the average surface density of the enzyme complexes to be one per 350 nm^2.

Proteoliposomes prepared with formate dehydrogenase complex alone were found to have a similar electron microscopic appearance (Fig. 5) to those containing fumarate reductase complex (Fig. 4). They exhibit spheres on the outer surface that are linked to the bilayer by means of stalks. From the diameter (7.5 nm) the molecular weight of the spheres is calculated as 181000.

Discussion

Dialysis of a solution of the two enzyme complexes in the presence of phospholipid, vitamin K-1 and octylglucoside led to a mixture of liposomes and small protein-phospholipid complexes. Upon freeze-thawing these complexes fused with the liposomes to form proteoliposomes. Density gradient centrifugation showed that these proteoliposomes were homogeneous in density. Their mean diameter was about 100 nm. The proteoliposomes were essentially unilamellar as shown by electron microscopy. The unilamellar structure was confirmed by the finding that the diameter calculated from the internal volume per g phospholipid was consistent with that obtained from electron microscopy (Table II). The particles on the surface of the proteoliposomes may be identified as the enzymes, since their size, orientation towards the outside, and surface density were consistent with those of the enzymes.

From the dimensions of the particles on the proteoliposomes prepared with fumarate reductase alone a molecular weight of 119000 was calculated (Table II). This value is smaller than that of the whole complex (200000) and is close to the molecular weight of the fumarate reductase without cytochrome *b* (121000) [4]. This result suggests that the cytochrome *b* may be buried in the membrane. Consistent with this view is the finding that the cytochrome *b* of the fumarate reductase com-

TABLE II

COMPARISON OF STRUCTURAL DATA ON THE PROTEOLIPOSOMES OBTAINED FROM ELECTRON MICROSCOPY AND BY OTHER METHODS

	Electron microscopy	Other methods	Reference
Inner diameter of the proteoliposomes (nm)	100	50–100	This paper, Results
Molecular weight of fumarate reductase	119000	121000	4
Molecular weight of formate dehydrogenase	181000	220000	3
Orientation of the enzymes	outside	80% outside	This paper, Table I
Surface area/enzyme molecule (nm^2)	250–400	350	This paper, Results

TABLE III

COMPOSITION OF THE PROTEOLIPOSOMES IN COMPARISON TO THAT OF THE CYTOPLASMIC MEMBRANE OF *V. SUCCINOGENES*

The data given for the bacterial membrane were calculated from the contents on a protein basis [3,4,6], using a protein/phospholipid ratio in the membrane of 3 g/g. The composition of the proteoliposomes was calculated from the amounts of the constituents used for preparation. The molecular weight of the enzyme complexes used was 200000 and that of phospholipid 1000. The values in the third column were calculated from those in the second one and refer to a proteoliposome with 100 nm diameter. A surface area of 0.7 nm^2 [8] was assumed to be occupied by a phospholipid molecule.

Constituent	Contents in		
	Bacterial membrane (mmol/mol phospholipid)	Proteoliposomes	
		mmol/mol phospholipid	Molecules/proteoliposome
Phospholipid	1000	1000	10^5
Menaquinone, vitamin K-1	15	10	1000
Fumarate reductase	2	0.1–1 [1]	10–100
Formate dehydrogenase	0.2	0.1–1 [1]	10–100

plex is a hydrophobic protein, whereas fumarate reductase lacking cytochrome *b* is essentially hydrophilic [2,4]. Furthermore, the high-potential cytochrome *b* of the fumarate reductase complex was recognized earlier as the direct electron acceptor from menaquinone or vitamin K-1 which are lipophilic and situated within the membrane [2,4]. Thus, the idea that the high-potential cytochrome *b* forms the membrane-binding site of fumarate reductase complex [2] is confirmed here by electron microscopy.

The formate dehydrogenase complex also consists of a hydrophilic and a lipophilic part. The hydrophilic part is represented by the spheres which are seen on the outer surface of proteoliposomes containing formate dehydrogenase alone. It is possible that the sphere represents the dimer of the bigger subunit (M_r 110000) which was previously proposed to constitute the formate dehydrogenase part of the complex [3]. In agreement with this possibility, the molecular weight of the sphere (181000) is not very different from that of the dimer (Table II).

The molar contents, based on phospholipid, of the quinone and enzyme complexes in the proteoliposomes are of the same order of magnitude as those in the cytoplasmic membrane of *V. succinogenes* (Table III). In the bacterial membrane the ratio of the contents of menaquinone, fumarate reductase and formate dehydrogenase is approx. 100 : 10 : 1. Similarly, in proteoliposomes a 10-fold molar excess of vitamin K-1 over the enzyme contents was required for maximal electron-transport activity [1]. The ratio of the levels of the enzymes in the proteoliposomes could be varied, however, from 0.1 to 10. Independently of this ratio, each enzyme molecule was found to participate in the electron transport [1]. Depending on the ratio of the enzyme contents, a proteoliposome of 100 nm diameter, consisting of 0.2 g protein/g phospholipid, was calculated to contain 10–100 molecules of each enzyme (Table III).

Acknowledgements

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