Electron transport driven phosphorylation catalyzed by proteoliposomes containing hydrogenase, fumarate reductase and ATP synthase

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Liposomes containing the hydrogenase complex and the fumarate reductase complex isolated from *Wolinella* (formerly *Vibrio*) *succinogenes*, together with vitamin K_1 catalyzed the electron transport from H_2 to fumarate. With the fumarate reductase complex present in excess the activity of electron transport was close to that of the hydrogenase complex. Liposomes containing an ATP synthase in addition to the electron transport components catalyzed the phosphorylation of ADP driven by the electron transport.

Electron transport *Phoshorylation* *Liposome* *Fumarate reductase* *Hydrogenase*

1. INTRODUCTION

The electron transport reaction (a) was earlier shown to be coupled

H_2 + fumarate $\longrightarrow$ succinate (a)

to the phosphorylation of ADP by inorganic phosphate in *Wolinella* (formerly *Vibrio*) *succinogenes* [1,2]. Aiming at an understanding of the mechanism of energy transduction, we describe here the reconstitution in liposomes of the electron transport chain catalyzing reaction (a). The capability of energy transduction of the reconstituted chain was tested using proteoliposomes containing an ATP synthase in addition to the electron transport components. The results will be discussed on the basis of the mechanism of energy transduction proposed earlier [3].

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, reduced DMN; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazone; Mops, 3-(*N*-morpholino) propanesulfonate

2. EXPERIMENTAL

2.1. *Preparation of proteoliposomes*

A lipid film consisting of 10 mg phosphatidylethanolamine (from *Escherichia coli*, sigma no. P 6398) and 0.2 μmol vitamin K_1 was prepared in a test tube [4], and dissolved in 2 ml of a buffer (pH 7.5, 0°C) containing 2 mg octylglucoside, 10 mM Mops, 0.25 M sucrose, 0.25 mM EDTA, hydrogenase complex (0.4 mg protein) and fumarate reductase complex (0.8 mg protein) which were isolated from *W. succinogenes* [4,5]. The solution was dialyzed for 20–24 h with two 1-l vols of an anaerobic buffer (pH 7.5, 0°C) containing 10 mM Mops, 2.5 mM $MgSO_4$, 1 mM malonate and 1 mM dithiothreitol. After dialysis, ATP synthase isolated from *W. succinogenes* (0.7 mg protein) (unpublished) was added. The mixture was frozen in liquid N_2 and subsequently thawed at room temperature. This procedure was repeated three times.

2.2. *Protein assay*

Protein was measured using the radioactivity which had been incorporated during growth of *W. succinogenes* in the presence of [^{3}H]leucine [6,7].

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3. RESULTS

3.1. *Structural properties of the proteoliposomes*

The preparation procedure described in section 2 led to the complete incorporation of the fumarate reductase complex and of the ATP synthase, while 40% of the hydrogenase complex could be separated from the proteoliposomes by sucrose density gradient centrifugation (not shown). Measurement of the fumarate reductase and ATPase activities before and after lysis [3,8] of the proteoliposomes after gradient centrifugation indicated that 60–70% of the enzymes were oriented to the outside of the vesicles. The orientation of the hydrogenase could not be measured.

The internal volume of the proteoliposomes was determined as 6.5 ml/g phospholipid. This value was calculated from the amounts of $[^3H]H_2O$ and [^{14}C]taurine which were sedimented together with the proteoliposomes from a suspension containing these compounds [3]. From the internal volume and the area occupied by a single phospholipid molecule (0.7 nm^2) the average diameter of the proteoliposomes was calculated as 0.2 μm [8]. Considering the enzyme/phospholipid ratio of the proteoliposomes, the average proteoliposome was found to contain 50 molecules of each enzyme or more.

3.2. *Electron transport*

The activities of electron transport ($H_2 \longrightarrow$ fumarate) of proteoliposomes prepared with or without the menaquinone analogue vitamin K_1 were compared to those of the hydrogenase and the fumarate reductase complex (table 1). The overall activity was slightly smaller than that of the limiting enzyme ($H_2 \longrightarrow$ DMN) with proteoliposomes containing vitamin K_1. In the absence of vitamin K_1 the activity of electron transport was more than 20 times smaller. These results suggested that: (i) the electron transport activity of the proteoliposomes was dependent on the presence of a lipophilic naphthoquinone, similar to the situation with the bacterial membrane [9]; and (ii) most, if not all, of the incorporated enzyme molecules participated in the electron transport reaction [3].

The hydrogenase complex used here catalyzed the reduction of the water-soluble menaquinone analogue DMN by H_2, while the hydrogenase isolated earlier did not [5]. Gel electrophoresis in the presence of dodecylsulfate indicated that the hydrogenase complex contained an additional third polypeptide of 58 kDa.

Table 1

Activities of electron transport, hydrogenase complex and fumarate reductase complex in proteoliposomes with and without vitamin K_1

Activity of	Proteoliposomes	
	Containing vitamin K_1 (U/mg total protein)	Without vitamin K_1 (U/mg total protein)
Electron transport ($H_2 \longrightarrow$ fumarate)	5.5	<0.2
Hydrogenase complex ($H_2 \longrightarrow$ DMN)	6.5	5.4
Fumarate reductase complex ($DMNH_2 \longrightarrow$ fumarate)	36.5	32.1

The proteoliposomes contained (in mg protein/g phospholipid) hydrogenase complex (41), fumarate reductase complex (85), and ATP synthase (70). The enzymic activities were measured photometrically at 270–290 nm using the absorbance of fumarate ($\Delta\epsilon$ = 0.45 $mM^{-1} \cdot cm^{-1}$) or DMN ($\Delta\epsilon$ = 16 $mM^{-1} \cdot cm^{-1}$). The proteoliposomes were suspended in 10 mM glycylglycine (pH 8.1), 37°C in a stoppered 0.5-cm cuvette. The suspensions were evacuated and flushed with N_2 ($DMNH_2 \longrightarrow$ fumarate) or H_2. The reactions were started by addition of 1 mM fumarate ($H_2 \longrightarrow$ fumarate or $DMNH_2 \longrightarrow$ fumarate). $DMNH_2$ (0.2 mM) was formed from DMN on the addition of KBH_4. $H_2 \longrightarrow$ DMN was started by adding 0.2 mM DMN. One unit of enzymic activity (U) was equivalent to the oxidation of 1 μmol H_2 or the reduction of 1 μmol fumarate per min

3.3. *Phosphorylation*

In the experiment shown in table 2 the proteoliposomes were incubated with H_2, labeled phosphate, hexokinase and glucose. The electron transport was started by the addition of fumarate. After 20 min (exp.1) or 10 min (exp.2), $HClO_4$ was added and organic phosphate and fumarate were measured. In control experiments a protonophore (FCCP) was added or fumarate was left out. The amount of organic phosphate formed was distinctly greater with fumarate present and in the absence of the protonophore, and both control values were identical. This suggested that the esterification of

Table 2

Formation of organic phosphate on incubation of proteoliposomes under phosphorylation conditions with H_2 and fumarate

Exp.	Assay	Fumarate consumed	Organic phosphate formed	P/fumarate
		(nmol)		
1	Complete	200	58	0.11
	Plus 30 μmol FCCP/g protein	200	36	–
	Without fumarate	0	36	–
2	Complete	4800	78	0.011
	Plus 30 μmol FCCP/g protein	4800	25	–

Proteoliposomes (0.21 mg total protein) were suspended in a medium (1 ml, pH 8, 37°C) containing 25 mM Hepes, 50 mM glucose, 2 mM [^{32}P]phosphate (8×10^3 Bq), 2 mM ADP, 2 mM $MgSO_4$, 2.3 mg bovine serum albumin, 30 units hexokinase and 1 mM dithiothreitol in 10-ml serum bottles. The suspension was evacuated for 20 min, then flushed with H_2 and agitated using a vertical shaker. Fumarate was added after 30 min (exp.1) or 25 min (exp.2) and $HClO_4$ after 50 (exp.1), or 35 min (exp.2). Organic phosphate and fumarate were determined as described [2]

part of the phosphate was due to electron transport coupled phosphorylation of ADP. The blank amount of the organic phosphate formed was probably caused by the incorporation of labeled phosphate into ADP. This reaction was found to be catalyzed by the isolated ATP synthase with the same specific activity (not shown). In contrast, the energy-dependent reactions of this enzyme required incorporation into liposomes.

The amount of organic phosphate formed by coupled phosphorylation increased linearly with the time of incubation (not shown), and was more than 2 orders of magnitude greater than that of the ATP synthase present. This showed that the reaction was not merely due to a single turnover of the enzyme. The phosphorylation yield (P/fumarate) was a function of the velocity of the electron transport (table 2) which was controlled by the degree of activation of the hydrogenase complex. The activity of this enzyme increased with the time of anaerobic incubation with H_2. The higher phosphorylation yield (0.11 mol phosphate/mol fumarate) was measured at 2% of the maximal rate of electron transport (exp.1). At the maximal rate of electron transport the yield was 10 times smaller (exp.2). The yield measured with a preparation of the bacterial membrane did not exceed 0.5 mol phosphate/mol fumarate [2].

4. DISCUSSION

4.1. *Reconstituted phosphorylation*

Proteoliposomes containing the two electron transport enzymes, hydrogenase complex and fumarate reductase complex, together with the ATP synthase of *W. succinogenes* catalyzed phosphorylation driven by reaction (a). The ATP synthase from spinach chloroplasts [10] or *Escherichia coli* [11] could replace the *Wolinella* enzyme. However, the yields and the rates were lower.

The ATP synthase of *W. succinogenes* was isolated using detergent extraction of the bacterial membrane (to be published). The enzyme (420 kDa) resembled those isolated from aerobic organisms in electron micrographs after negative staining and consisted of 6 different subunits, 3 of which made up the hydrophilic ATPase part. The ATP synthase activity was inhibited by DCCD which bound specifically to the smallest subunit of the lipophilic part.

The turnover numbers of the externally oriented

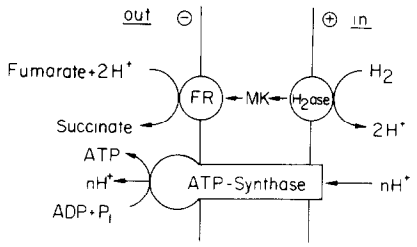

Fig.1. Proposed mechanism of energy transduction in the electron transport coupled phosphorylation catalyzed by the proteoliposomes containing hydrogenase complex (H_2 ase), fumarate reductase complex (FR) and ATP synthase.

ATP synthase molecules in the experiment of table 2 corresponded to about 1% of that measured with the enzyme in the bacterial membrane [2]. The turnover number increased from 13 to 62 min^{-1}, when the activity of electron transport increased by a factor of 47. This suggested that the phosphorylation reaction was limited by the activity of the ATP synthase in the proteoliposomes. Experiments with liposomes containing the ATP synthase alone (not shown) supported this view. The rate of ATP synthesis was a function of the magnitude of the $\Delta\tilde{\mu}_H$ which was applied across the liposomal membrane to drive the reaction. The turnover number was in the order of magnitude of those of table 2 at values of $\Delta\tilde{\mu}_H$ below 150 mV, and approached that of the bacterial membrane at $\Delta\tilde{\mu}_H$ = 200 mV. The $\Delta\tilde{\mu}_H$ generated by electron transport was nearly 100 mV (positive inside) with the proteoliposomes used here.

4.2. *Mechanism of energy transfer*

As a working hypothesis it was earlier proposed that the free energy of reaction (a) was transferred to the phosphorylation reaction by means of a $\Delta\tilde{\mu}_H$ which could be generated by transmembrane electron transport (fig.1) [3,12]. This hypothesis was supported here by the finding that phosphorylation, driven by reaction (a), required only the 2 electron transport complexes and an ATP synthase. Consistent with the mechanism, the $\Delta\tilde{\mu}_H$ generated across the liposomal membrane by reaction (a) was inside positive. Proteoliposomes containing the formate dehydrogenase instead of the hydrogenase complex, catalyzed reaction (b) [4,8,13] and did neither

formate + fumarate ⟶ CO_2 + succinate (b)

generate a $\Delta\tilde{\mu}_H$ on initiation of the electron transport nor catalyze coupled phosphorylation (not shown). This is in agreement with the mechanism of fig.1, since formate, in contrast to H_2, does not penetrate through the membrane and can therefore react only with the formate dehydrogenase molecules that face the outside of the proteoliposomes [3,8]. As a consequence, a $\Delta\tilde{\mu}_H$ is not expected to be generated.

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