NADH oxidation in phospholipid-enriched cytoplasmic membrane vesicles from *Escherichia coli*

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Received 14 March 1983

NADH oxidation in *Escherichia coli* cytoplasmic membrane vesicles enriched in anionic phospholipids by de novo synthesis of lipid in the vesicles from acyl-CoA esters and *sn*-glycerol 3-phosphate has been studied. NADH-oxidase but not NADH-dehydrogenase activity was found to decrease during synthesis and accumulation of phospholipid in the vesicles. Density gradient fractionation showed that NADH-oxidase activity was reduced to ~30% in vesicles with a 3–6-fold increase in anionic phospholipid, whereas vesicles with a >10-fold increase in phospholipid had virtually no NADH oxidase activity.

Bacterial membrane *Anionic phospholipid* *Respiratory chain* Escherichia coli

1. INTRODUCTION

Cytoplasmic membrane vesicles from *Escherichia coli* synthesize substantial amounts of the anionic phospholipids phosphatidylglycerol, cardiolipin and phosphatidic acid when they are supplied with *sn*-glycerol 3-phosphate, CTP and acyl-CoA esters under proper conditions [1]. The newly-formed phospholipid is incorporated in the vesicle membrane and the density of the vesicles decreases accordingly [1,2]. The present investigation was undertaken to explore the possibility, that such lipid-enriched vesicles could be useful in studies of protein–lipid relations in biological membranes.

The bacterial respiratory chain makes up an essential part of the protein in the *E. coli* cytoplasmic membrane [3] and is likely to depend for its activity on the composition and amount of lipid in the membrane as has been found for its eukaryotic counterpart in the mitochondrial inner membrane [4,5]. The oxidation of NADH in the lipid-enriched vesicles was therefore examined. It was found that NADH-oxidase but not NADH-dehydrogenase activity was reduced in the phospholipid-enriched vesicles and almost completely abolished when the enrichment was >10-fold.

2. MATERIALS AND METHODS

2.1. *Bacterial strain and preparation of cytoplasmic membrane vesicles*

Cytoplasmic membrane vesicles were made by sonication of spheroplasts prepared from *E. coli* K-12 cells (strain 58-161), which were harvested in the early stationary phase after aerobic growth at 37°C [2].

Enrichment of native vesicles in anionic phospholipids was achieved by de novo phospholipid synthesis from acyl-CoA esters and *sn*-glycerol 3-phosphate in the presence of CTP. The conditions were defined as in [1] except that the following modifications were introduced:

(i) In order to diminish aggregation of vesicles, which caused a pronounced cloudiness of the vesicle suspensions in the later stages of incubation, 0.25 M sucrose was included in the incubation medium;

(ii) Unspecific detergent-like effects of acyl-CoA [6] were prevented by bovine serum albumin, which was included in the acyl-CoA solutions

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00145793/83/$3.00

continuously infused in the incubation mixture.

Determination of free acyl-CoA during the incubation and yield of phospholipid showed, that the concentration of free acyl-CoA would not exceed 20% of the added amount at any time during incubation. This concentration of acyl-CoA was shown in independent experiments (not shown) not to exert any direct inhibitory effect on NADH-oxidase activity of the vesicles provided that fatty acid-free serum albumin was present in a 0.5 molar ratio to the acyl-CoA. Albumin was therefore included in the acyl-CoA solutions in a molar ratio of 0.1 to acyl-CoA.

Control vesicles were prepared by the same procedure as lipid-enriched vesicles except that acyl-CoA was omitted from the serum albumin solution fed to the incubation mixture.

Vesicles were recovered from the incubation mixture by centrifugation at 100000 × *g* for 1 h. They were resuspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 8.0), frozen in liquid N_2 and stored at −70°C.

2.2. *Assay of NADH oxidation*

NADH-oxidase activity was assayed either spectrophotometrically or with an oxygen electrode. NADH-dehydrogenase activity was assayed spectrophotometrically with ferricyanide (1 mM) as electron acceptor in the presence of 0.14% Triton X-100, which abolished oxygen consumption completely. The assay media were 150 mM KCl, buffered with 10 mM K-phosphate (pH 7.4) at 24°C.

2.3. *Other methods*

Sucrose gradient centrifugation, phospholipid analysis and protein determinations were made as in [1] and polyacrylamide gel electrophoresis was performed as in [7]. Fatty acid-free serum albumin was prepared from crystalline bovine serum albumin as in [8].

3. RESULTS AND DISCUSSION

Table 1 summarizes data from phospholipid analysis and assays of NADH oxidation in native, lipid-enriched and control *E. coli* cytoplasmic membrane vesicles. The lower activities in control vesicles compared to native vesicles show that incubation followed by ultracentrifugation and freezing of vesicles itself gave rise to inactivation of NADH-dehydrogenase and NADH-oxidase activity. It is clear, however, that the NADH-oxidase activity was markedly lower in the lipid-enriched vesicles than in the control, whereas the NADH-dehydrogenase activity was the same in the two vesicle preparations.

The time-course of inactivation of NADH-oxidase activity during incubation with acyl-CoA infusion and phospholipid synthesis is shown in fig.1. In contrast to the linear increase in phospholipid [1], inactivation was initially rapid but the rate decreased within the first hour to a value which was only slightly higher than the rate in the control incubation.

A population of vesicles with a low capacity for de novo synthesis of phospholipid is known to be produced during disruption of *E. coli* spheroplasts [1]. If these vesicles had retained their NADH-oxidase activity unimpaired during the incubation their activity could possibly account for the total activity measured in the vesicle suspension after in-

Table 1

Phospholipid content and NADH-oxidation in native, control and lipid-enriched cytoplasmic membrane vesicles from *E. coli*

Vesicle preparation	Phospholipid[a]		NADH-oxidase activity	NADH-dehydrogenase activity
	PE	PG + CL + PA	[μmol NADH ox..min^{-1}.mg protein^{-1} ± SD (n = 17)]	
	[nmol.mg protein^{-1} ± SD (n = 4)]			
Native	360 ± 61	190 ± 50	2.3 ± 0.4	1.6 ± 0.3
Control	250 ± 34	148 ± 38	0.9 ± 0.3	0.9 ± 0.5
Lipid-enriched	260 ± 25	860 ± 134	0.2 ± 0.1	0.9 ± 0.4

[a] PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid

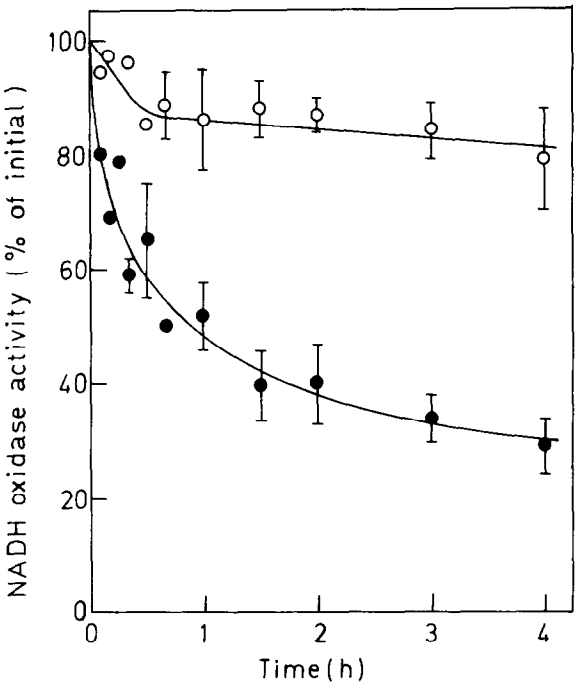

Fig.1. Time-course of inactivation of NADH-oxidase activity in cytoplasmic membrane vesicles from *E. coli* during de novo synthesis of phospholipid. Samples of 50 μl were taken from the incubation mixture and assayed for NADH-oxidase activity: from incubation (●) with acyl-CoA; and (○) without infusion.

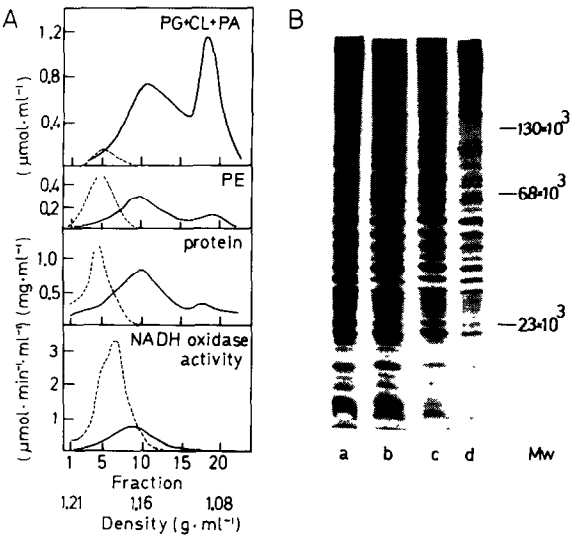

Fig.2. Density gradient fractionation of control and lipid-enriched cytoplasmic membrane vesicles from *E. coli*: (A) anionic phospholipids, phosphatidylethanolamine, protein and NADH-oxidase activity profiles. Abbreviations as in table 1. Control (– – –), lipid-enriched (——); (B) SDS–polyacrylamide gel electrophoresis of native vesicles (a) and of peak fractions from control (b) and lipid-enriched preparations: (c) high density and (d) low-density vesicles. β-Galactosidase, bovine serum albumin and bovine chymotrypsin were used as M_r markers.

cubation. After incubation the vesicles were therefore separated by density gradient centrifugation according to lipid/protein ratio and then assayed for NADH oxidase activity.

The newly-formed anionic phospholipid banded together with phosphatidylethanolamine and protein in two separate density regions indicating the existence of a heavier and a lighter vesicle population (fig.2). The residual NADH-oxidase activity was found almost exclusively in association with the heavier vesicles in which the anionic phospholipid had increased only 3–6-fold. In the lighter vesicles where little if any NADH-oxidase was present the increase in phospholipid was 10–20-fold. No qualitative differences in protein composition between light and heavy vesicles could be demonstrated. The same number and location of protein bands was found in SDS–gel electrophoresis of peak fractions from the two density regions (fig.2B).

Results from 3 density gradient fractionations are summarized in table 2. They demonstrate a clear correlation of NADH-oxidase activity with the ratio of phosphatidylethanolamine to anionic phospholipid. It also appears, however, that the respiratory chain retains 25–30% of its activity in the heavier of the lipid-enriched vesicles despite a complete reversal of the proportions of phosphatidylethanolamine and anionic phospholipid in the membrane. This is in agreement with the results in [9] where little difference was found in NADH-oxidase activity of cytoplasmic membrane vesicles prepared from wild type *E. coli* and from a mutant with a 2–3-fold higher content of anionic phospholipid in the membrane. Since growth of *E. coli* is known to be inhibited already when the amount of anionic phospholipid exceeds 40–70% of the total membrane lipid [9], the above results suggest that this growth inhibition is not primarily a result of a

Table 2

Phospholipid content and NADH-oxidase activity in peak fractions[a] after density gradient centrifugation of control and lipid-enriched cytoplasmic membrane vesicles from *E. coli*

Vesicle preparation	Density of peak	Phospholipid[b]			NADH-oxidase activity (μmol NADH.min^{-1}. mg protein^{-1})
		PE (nmol.mg protein^{-1})	PG+CL+PA	$\frac{PE}{PG+CL+PA}$	
Control ($n = 3$)	1.16–1.19	592 ± 225	212 ± 36	2.8	4.2 ± 0.3
Lipid-enriched ($n = 3$)	1.14–1.17	475 ± 68	876 ± 427	0.5	1.1 ± 0.3
	1.08–1.12	530 ± 106	3826 ± 2021	0.1	0.2 ± 0.1

[a] Average values from peak fraction and fraction before and after
[b] PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid

decreased function of the respiratory chain.

The study has thus shown that relevant information on protein–lipid relations in membranes may be obtained by the use of *E. coli* cytoplasmic membrane vesicles in which the phospholipid content has been modified by de novo synthesis in vitro. A number of related studies (such as the diffusional mobility of proteins in membranes [5,10]) could profit from the use of such vesicles, as the modification of phospholipid may be partially governed by manipulation of the in vitro conditions [11].

ACKNOWLEDGEMENTS

E.J.F.D. is grateful to the Danish Cancer Society and the Danish Medical Research Council for financial support during the present study. We thank Mrs Ellen Philipson and Mr John Svaneborg for technical assistance.

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