Comparison of the effects of one-side- and two-side Mg^{2+} on the reconstitution of mitochondrial H^+-ATPase

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Three types of proteoliposome containing mitochondrial H^+-ATPase have been prepared: Mg^{2+}-'free', one-side and two-side Mg^{2+}-containing proteoliposomes. The ATPase activity as well as its sensitivity to oligomycin or *N,N'*-dicyclohexylcarbodiimide of the three proteoliposome preparations has been compared. They decreased in the order: $L \cdot (H^+\text{-ATPase})_{+Mg^{2+}} > L \cdot (H^+\text{-ATPase})_{\mp Mg^{2+}} > L \cdot (H^+\text{-ATPase})_{-Mg^{2+}}$. The fluidity of the proteoliposomes has also been compared by fluorescence polarization probes diphenylhexatriene (DPH) or 7-(9-anthroyloxy)stearic acid (7-AS). The degree of polarization for DPH in these proteoliposomes decreased in the order: $L \cdot (H^+\text{-ATPase})_{+Mg^{2+}} > L \cdot (H^+\text{-ATPase})_{\mp Mg^{2+}} > L \cdot (H^+\text{-ATPase})_{-Mg^{2+}}$, while that for 7-AS: $L \cdot (H^+\text{-ATPase})_{+Mg^{2+}} \approx L \cdot (H^+\text{-ATPase})_{\mp Mg^{2+}} > L \cdot (H^+\text{-ATPase})_{-Mg^{2+}}$.

Lipid fluidity *Mitochondrial H^+-ATPase* *One-side Mg^{2+} effect* *Two-side Mg^{2+} effect* *Lipid-protein interaction*

1. INTRODUCTION

We have reported [1,2] that during reconstitution of porcine heart mitochondrial H^+-ATPase in soybean phospholipid liposomes by the cholate dialysis method, Mg^{2+} greatly enhances [^{32}P]ATP exchange activity, ATPase activity, and its sensitivity to oligomycin in reconstituted proteoliposomes. The effect of Mg^{2+} on the fluidity of the reconstituted proteoliposomes was measured by means of spin label probes, 5-NS, 12-NS and 16-NS. The results obtained showed that Mg^{2+} may cause a change in fluidity of the lipid molecules near the surfaces of the bilayer, but does not significantly affect the deeper layer of the reconstituted systems. The conformations of the Mg^{2+}-containing and the Mg^{2+}-'free' proteoliposomes were compared by studying circular dichroism spectra. The α-helical content of the Mg^{2+}-containing proteoliposomes was higher. Therefore, we postulate that Mg^{2+} may play a role in altering the fluidity of the proteoliposomes, which would favor the formation of a conformation of the reconstituted H^+-ATPase with higher activity.

It was noticed that the effect of Mg^{2+} on the incorporation of the H^+-ATPase complex depends on the method of reconstitution. It was more evident in the cholate dialysis method than in the incubation method. Presumably, this may be due to a simultaneous Mg^{2+} effect on both sides of the lipid bilayer in cholate dialysis. Here, one-side and two-side Mg^{2+} effects on the reconstitution of mitochondrial H^+-ATPase-containing proteoliposomes have been compared.

Abbreviations: 5-NS, 12-NS, and 16-NS, the 5-, 12- and 16-(*N*-oxyl)-4',4'-dimethyloxazolidine derivatives of stearic acid, respectively; 7-AS, 7-(9-anthroyloxy)stearic acid; DCCD, *N,N*-dicyclohexylcarbodiimide; DPH, diphenylhexatriene

2. MATERIALS AND METHODS

Porcine heart mitochondrial H^+-ATPase was isolated by the method of Kagawa and Racker [3] as described in detail in the preceding paper [4].

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Three types of H^+-ATPase incorporating proteoliposomes were reconstituted as follows:

(i) Mg^{2+}-'free' proteoliposomes – $L\cdot(H^+\text{-ATPase})_{-Mg^{2+}}$

A suspension of 75 mg purified soybean phospholipid in 1 ml of a solution containing 10 mM Tris (pH 8.0), 0.2 mM EDTA, 0.64 mM dithiothreitol and 2% cholate (pH 8.0) was subjected to sonic oscillation. 0.8 ml of the solution containing 60 mg of the sonicated phospholipid plus 8 mg H^+-ATPase protein was dialysed against 300 vols of a solution containing 10% methanol, 5 mM mercaptoethanol, 0.2 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.1 mM Na_2ATP for 22 h at 0–4°C.

(ii) Two-side Mg^{2+}-containing proteoliposomes – $L\cdot(H^+\text{-ATPase})_{+Mg^{2+}}$

H^+-ATPase-incorporating proteoliposomes were reconstituted by the cholate dialysis method in the presence of 1 mM Mg^{2+} in the dialysis medium for 22 h at 0–4°C.

(iii) One-side Mg^{2+}-containing proteoliposomes – $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$

It was reported [3] that the concentration of cholate was very low after 16 h dialysis and the reconstitution was nearly complete by this time. Similar results were obtained in our laboratory. Since the phospholipid bilayer of vesicles is impermeable to divalent cations, the reconstitution of $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$ might be performed as follows: after about 16 h dialysis in the absence of Mg^{2+}, the reconstituted particles were dialysed for a further 6 h in the presence of 1 mM Mg^{2+}. In this case Mg^{2+} was present only in the outer phospholipid layer of the formed proteoliposomes.

3. RESULTS AND DISCUSSION

We reported [1] that a difference in fluidity seems to be located near the polar faces of the lipid bilayer of the Mg^{2+}-containing and the Mg^{2+}-'free' proteoliposomes. Here, two probes of fluorescence polarization, DPH and 7-AS, were used separately to measure and compare the fluidity of $L\cdot(H^+\text{-ATPase})_{-Mg^{2+}}$, $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$ and $L\cdot(H^+\text{-ATPase})_{+Mg^{2+}}$. DPH is hydrophobic and its degree of fluorescence polarization may reflect the average motion and viscosity of lipid molecules in both layers of the proteoliposomes. From table 1 it can be seen that the degree of polarization decreased in the order: $L\cdot(H^+\text{-ATPase})_{+Mg^{2+}} > L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}} > L\cdot(H^+\text{-ATPase})_{-Mg^{2+}}$. 7-AS was also used to compare the fluidity of the three types of proteoliposomes. As an amphipathic molecule it can only be intercalated in the outer phospholipid layer of the proteoliposomes. From the 7-AS fluorescence polarization measurements it is interesting to note that, contrary to the results obtained using DPH, there was almost no difference between the degree of polarization of $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$ and that of $L\cdot(H^+\text{-ATPase})_{+Mg^{2+}}$. But the degree of polarization of $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$ was still higher than that of

Table 1

Fluorescence polarization (P) of DPH or 7-AS in the three types of proteoliposome preparations

Proteoliposome preparation	Fluorescence polarization (P)		t-test			
	DPH	7-AS	Comparison vs $L\cdot(H^+\text{-ATPase})_{-Mg^{2+}}$		Comparison vs $L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$	
			DPH	7-AS	DPH	7-AS
$L\cdot(H^+\text{-ATPase})_{-Mg^{2+}}$	0.150 ± 0.002 (6)	0.153 ± 0.001 (4)				
$L\cdot(H^+\text{-ATPase})_{\mp Mg^{2+}}$	0.158 ± 0.001 (6)	0.161 ± 0.002 (4)	$P < 0.01$	$P < 0.05$		
$L\cdot(H^+\text{-ATPase})_{+Mg^{2+}}$	0.164 ± 0.001 (6)	0.162 ± 0.001 (4)			$P < 0.01$	ns

Fluorescence polarization was measured at 25°C, in 2 ml of medium containing 10 mM Tris-HCl (pH 8.0), about 200 μg phospholipid of proteoliposomes and 1×10^{-3} μmol DPH or 7-AS were added. The mixture was incubated at 25°C for 40 min. The excitation and emission wavelengths were 360 and 428 nm for DPH, 365 and 440 nm for 7-AS, respectively. Values of fluorescence polarization (P) are the mean value ± SE with the number of experiments in parentheses. ns, not significant

Table 2

ATPase activity of L·(H^+-ATPase) reconstituted in the presence or absence of Mg^{2+}

Proteoliposome preparation	ATPase activity and its sensitivity to inhibitors					
	Activity		Oligomycin		DCCD	
	Activity increase (%)		Activity inhibition (%)		Activity inhibition (%)	
L·(H^+-ATPase)$_{-Mg^{2+}}$	3.89	–	3.19	18	3.54	9
L·(H^+-ATPase)$_{\mp Mg^{2+}}$	4.69	21	2.63	44	3.71	21
L·(H^+-ATPase)$_{+Mg^{2+}}$	7.76	99	2.41	69	5.04	35

The ATPase activity was measured at 30°C for 5 min, in a medium containing about 30 μg enzyme protein, 20 mM Tris-H_2SO_4 (pH 8.0), 2 mM $MgSO_4$, 50 mM KCl, 0.5 mM EDTA, 20 μg pyruvate kinase, 1 μmol phospho*enol*pyruvate and 2.5 μmol Na_2ATP. To the medium 0.4 μg oligomycin or DCCD was added for the inhibition assay. The enzyme activity is expressed as μmol P_i/mg protein per 7 min

L·(H^+-ATPase)$_{-Mg^{2+}}$. This suggests that in the one side-Mg^{2+}-containing proteoliposomes only the fluidity of the outer layer has been changed.

The ATPase activity as well as its sensitivity to oligomycin or DCCD of L·(H^+-ATPase)$_{-Mg^{2+}}$, L·(H^+-ATPase)$_{\mp Mg^{2+}}$ and L·(H^+-ATPase)$_{+Mg^{2+}}$ was compared. The results showed that both the one-side and two-side Mg^{2+} effect could markedly increase the ATPase activity as well as its sensitivity to oligomycin or DCCD of the reconstituted enzyme complex, but the simultaneous Mg^{2+} effect on both sides of the lipid bilayer in cholate dialysis was more marked.

It is likely that a proper physical state of surrounding lipid is necessary for membrane proteins embedded in the lipid bilayer, allowing them to ensure a suitable conformation for activity. The H^+-ATPase complex is known to consist of two main components: the hydrophobic protein (F_0), which is buried in the lipid core and the soluble ATPase (F_1). It was postulated that the conformation of F_0 buried in the hydrophobic portion depends on the physical state of the surrounding phospholipid molecules [5]. The conformation of the F_1 will be influenced in turn by the conformational change of F_0 [6]. The present results show that the lipid packing near the surface of the outer layer of the proteoliposomes could be altered by the one side-Mg^{2+} effect, which might partly induce the conformational change of the incorporated F_0 of the H^+-ATPase complex and hence increase the ATPase activity as well as its sensitivity to oligomycin or DCCD. However, this effect is not so clear as in the case of two-side Mg^{2+}-containing proteoliposomes. Presumably, the simultaneous Mg^{2+} effect may offer both in the outer and inner layer a proper physical state of the phospholipids, favouring the formation of a more suitable conformation of the H^+-ATPase with higher enzyme activity.

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