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ROLE OF Mg^{2+} IN LIPID-PROTEIN INTERACTION IN RECONSTITUTED PORCINE HEART MITOCHONDRIAL H^+-ATPase

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During reconstitution of pig heart mitochondrial H^+-ATPase in soybean phospholipid liposomes by the cholate dialysis method, Mg^{2+} greatly enhances $^{32}P_i$-ATP exchange activity, ATPase activity and the sensitivity to oligomycin of the reconstituted enzyme complex. The effect of Mg^{2+} on the fluidity of the reconstituted proteoliposomes was measured by means of a fluorescent probe, 1-anilinonaphthalene-8-sulfonate, and spin-label probes, 5-nitroxide stearate, 12-nitroxide stearate and 16-nitroxide stearate. A difference in fluidity seems to be localized near the polar faces of the lipid bilayers of the reconstituted proteoliposomes. Fluidity was less in the presence of Mg^{2+} than in its absence. 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. 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.

Introduction

In recent years, the reconstitution of a number of membrane proteins (including enzymes) in liposomes has been carried out in many laboratories [1–5], but the results have not always been easily reproducible. Sone et al. [5] reported that Mg^{2+} promotes the reconstitution of the H^+-ATPase complex (TF_0-F_1) from the thermophilic bacterium PS3 in lipid vesicles, when both deoxycholate and cholate were used to solubilize the membrane preparation. In the case of reconstitution of pig heart mitochondrial H^+-ATPase in soybean phospholipid liposomes, a marked Mg^{2+} effect was also observed when only cholate was used to solubilize the membranes [6].

What is the role of Mg^{2+}? It has been suggested that Mg^{2+} may affect the lipid environment of the reconstituted H^+-ATPase in the proteoliposomes, thus changing its conformation and increasing its activity [6]. In the present study, we have investigated the effect of Mg^{2+} on the fluidity of the phospholipids of H^+-ATPase proteoliposomes by observing the fluorescence of the fluorescent probe anilinonaphthalenesulfonate and the ESR spectrum using various spin label probes: 5-NS, 12-NS and 16-NS. In addition, we have observed the effect of Mg^{2+} on the conformation of incorporated H^+-ATPase by means of circular dichroism (CD) studies. The results suggest that Mg^{2+} alters the fluidity of the lipid matrix, leading to a conformational change in the reconstituted H^+-ATPase, which increases its activity.

Abbreviations: DCCD, *N*,*N'*-dicyclohexylcarbodiimide; 5-, 12- and 16-NS, the 5-, 12- and 16-(*N*-oxyl)-4',4'-dimethyloxazolidine derivatives of stearic acid, respectively.

Materials and Methods

Preparation of H^+-ATPase from pig heart mitochondria. Pig heart mitochondrial H^+-ATPase was isolated by the method of Kagawa and Racker [1] as described in detail in the preceding paper [7].

Purification of soybean phospholipids. Crude soybean phospholipids (5 g) were dissolved in 25 ml acetone by stirring until the supernatant became clear. The insoluble residue was extracted four more times with the same volume of acetone. The residue was finally sedimented by centrifugation at $1000 \times g$ for 15 min. To the sediment 20 ml diethyl ether containing some tocopherol were added and the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant was concentrated to dryness under reduced pressure. The sediment was then dissolved and stored in chloroform/methanol (4 : 1) at $-20°C$.

Reconstitution procedure. Reconstitution was performed by means of the cholate dialysis method [1]. A suspension of 100 mg purified soybean phospholipids in 0.68 ml of a solution containing 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.3 mM dithiothreitol and cholate was subjected to sonic oscillation in an MSE ultrasonic disintegrator at 20 kHz and 150 W in an ice bath for 2 min. 1 ml of the solution containing 75 mg of the sonicated phospholipids, 10 mg H^+-ATPase protein and 2% cholate was dialyzed against 100 vol. 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 and 0–5 mM $MgCl_2$ for 20 h at 0–4°C

Analytical methods. $^{32}P_i$-ATP exchange activity was assayed according to the method of Conover et al. [8]. The activity is expressed as μmol P_i/mg per min.

The ATPase activity and its sensitivity to oligomycin and DCCD were assayed according to the method of Serrano et al. [4]. The enzyme activity is expressed as μmol P_i/mg per min.

Protein was determined according to the method of Lowry et al. [9], using bovine serum albumin as standard.

Fluorescence studies. The assay system consisted of 2.5 ml solution containing proteoliposomes (160–350 μg phospholipids), 25 μmol anilinonaphthalenesulfonate and 250 μmol Tris-H_2SO_4 (pH 7.4). Fluorescence of anilinonaphthalenesulfonate was measured in a MPF-4 spectrophotofluorometer at 25°C with an excitation wavelength of 380 nm and 472 nm emission wavelength.

ESR spectra. The spin label probes used were the 5-, 12- and 16-(*N*-oxyl)-4′,4′-dimethyloxazolidine derivatives of stearic acid (designated 5-NS, 12-NS and 16-NS), obtained from Syva (Plano, TX, U.S.A.). The spin-labeled samples were prepared as follows: to 2–3 μl of 0.25 mM 5-NS in $CHCl_3$ were added 50 μl of redistilled diethyl ether. The solvent was removed under vacuum and 10 μl of 50 mM $K_3Fe(CN)_6$, 20–30 μl proteoliposome solution (600–750 μg phospholipid) and 50 μl of 20 mM Tris-H_2SO_4 (pH 8.0) were added successively so as to make up a final volume of 0.1 ml. The solution was stirred for 10–15 min, and then placed in a sample tube. The ESR spectra were recorded in a 404 Type ESR spectrometer (built at the Institute of Biophysics, Academia Sinica) at room temperature. From the spectra, the distances of the inner and outer extremes of the hyperfine splitting ($2T_\perp$, $2T_\parallel$) were measured and the order parameter (S) was calculated [10]:

$$S = 0.568 \frac{T_\parallel - T_\perp}{a'} \qquad a' = \tfrac{1}{3}(T_\parallel + 2T_\perp)$$

The freedom of motion of spin labels in the membrane was calculated as the rotational correlation time (τ_c) by means of the following equation:

$$\tau_c = 6.5 \cdot 10^{-10} \times W_0 \left(\sqrt{\frac{h_0}{h_{-1}}} - 1 \right)$$

where W_0 is the linewidth of the medium field line, and h_0 and h_{-1} the heights of the medium- and high-field lines, respectively.

CD study. The CD spectra were obtained with a Dichrograph III instrument at 20°C. The measurements were carried out on 0.10 scale in a cell with 0.1 mm optical path length at a sensitivity of $2 \cdot 10^{-6}$/mm.

Results

Effect of Mg^{2+} on reconstitution of the H^+-ATPase with soybean phospholipid liposomes

The activities of the reconstituted H^+-ATPase

were significantly enhanced by the presence of Mg^{2+} in the dialysis medium and the results were easily reproducible. The $^{32}P_i$-ATP exchange activity and the sensitivity of the enzyme activity to oligomycin and DCCD were measured.

The $^{32}P_i$-ATP exchange activity of the enzyme reconstituted in the presence of 1 mM Mg^{2+} was increased about 6-fold compared with that reconstituted in the absence of Mg^{2+} (Table I). The maximal $^{32}P_i$-ATP exchange activity was obtained at a concentration of 1 mM Mg^{2+} in the dialysis medium. The optimal pH of the dialysis medium for proteoliposome formation with high exchange activity was pH 8.0–8.5.

The ATPase activity of the enzyme complex before reconstitution was very low, and was markedly increased by reconstitution. The activity could be stimulated a further 100% by addition of F_1 [6]. The presence of Mg^{2+} in the dialysis medium also markedly increased the ATPase activity of the reconstituted enzyme as well as its sensitivity to oligomycin (Table I). The ATPase activity was increased about 50% in the presence of 1 mM Mg^{2+}. Its sensitivity to oligomycin was increased by 113%, but the DCCD sensitivity was not significantly changed. These effects were not due to the increase in Mg^{2+} concentration in the assay medium by Mg^{2+} present in the proteoliposome preparation. Addition of the same amount of Mg^{2+} as that in Mg^{2+}-containing proteoliposomes to the assay medium did not significantly raise the ATPase activity of the proteoliposomes formed in the absence of Mg^{2+}.

Fluidity of Mg^{2+}-containing and Mg^{2+}-free proteoliposomes studied with a fluorescent probe

Amphipathic molecules like 1-anilinonaphthalene-8-sulfonate accumulate in the lipid bilayer at the lipid/water interface, where their fluorescence is sensitive to the viscosity of their microenvironment [11]. We have, therefore, used anilinonaphthalenesulfonate fluorescence studies to compare the fluidity of the membranes of the two proteoliposome preparations.

Fig. 1 displays a double-reciprocal plot of fluorescence intensity (F) versus the concentration of soybean phospholipid in liposomes (W_p). It clearly shows that the quantum yield of fluorescence in the Mg^{2+}-containing proteoliposomes is higher than in those not containing Mg^{2+}. Since the increase in fluorescence intensity is not accompanied by a shift of the wavelength of maximal emission, the viscosity or fluidity of the microenvironment of the anilinonaphthalenesulfonate probe, rather than the polarity of this microenvironment, appears to have been changed by the presence of Mg^{2+}. Addition of the same amount of Mg^{2+} present in the Mg^{2+}-containing proteoliposomes to the Mg^{2+}-free proteoliposomes does not change the fluorescence intensity. This is consistent with our finding that Mg^{2+} can bring about higher activity of the reconstituted H^+-ATPase

TABLE I

ENZYME ACTIVITIES OF H^+-ATPase RECONSTITUTED IN THE PRESENCE OR ABSENCE OF Mg^{2+}

$^{32}P_i$-ATP exchange was measured at 30°C for 15 min in a medium containing 20 μmol Tris-HCl (pH 8.0), 0.5 μmol $MgCl_2$, 10 μmol Na_2ATP, 2.5 mg bovine serum albumin and approx. 300 μg enzyme protein. The reaction was initiated by addition of 20 μmol $^{32}P_i$ ($2–3 \cdot 10^7$ cpm). It was terminated by adding 0.1 ml of 35% perchloric acid. The data are averages of five experiments. The ATPase activity was measured at 30°C for 5 min in a medium containing about 20 μg enzyme protein, 20 mM Tris-H_2SO_4 (pH 8.0), 2 mM $MgCl_2$, 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 0.8 μg oligomycin or 0.4 μg DCCD was added for the inhibition assay. The enzyme activity is expressed as μmol P_i/mg protein per min.

Proteoliposome preparation	$^{32}P_i$-ATP exchange activity		ATPase activity and its sensitivity to inhibitors					
			Activity	Increase (%)	Oligomycin		DCCD	
	Activity	Increase (%)			Activity	Inhibition (%)	Activity	Inhibition (%)
$-Mg^{2+}$	0.003	–	1.23	–	0.84	32	1.06	14
$+Mg^{2+}$	0.021	600	1.89	54	0.61	68	1.60	16

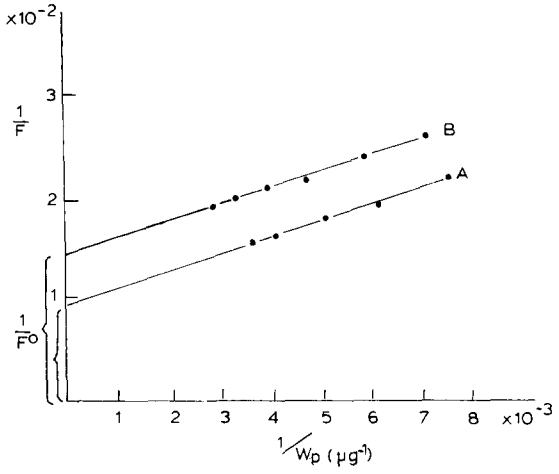

Fig. 1. Double-reciprocal plot of anilinonaphthalenesulfonate fluorescence intensity versus phospholipid content. (A) Mg^{2+}-containing proteoliposomes, (B) Mg^{2+}-free proteoliposomes. Samples of 2.5 ml assay medium containing proteoliposomes, 25 μmol anilinonaphthalenesulfonate and 250 μmol Tris-H_2SO_4 (pH 7.4) were incubated at 25°C for 5 min. The fluorescence intensity was measured at 25°C using 380 nm excitation and 472 nm emission.

only when the Mg^{2+} was present during reconstitution.

Fluidity of Mg^{2+}-containing and Mg^{2+}-free proteoliposomes studied with ESR spin labels

Spin-labeled stearic acids were also used to compare the fluidity of the two types of proteoliposomes. After introduction of the spin label in the proteoliposomes, its fatty acyl chain is tightly intercalated between the phospholipid fatty acyl chains while its polar group points toward the lipid/water interface. Spin labels containing the nitroxide group at varying positions along the acyl chain were used to assess the fluidity at varying depth in the lipid bilayers.

From the ESR spectra for the spin label 5-NS the outer and inner extremes of the hyperfine splitting (in gauss, denoted by $2T_{\parallel}$ and $2T_{\perp}$) were measured (Fig. 2) and the order parameters were calculated. Table II shows that the outer extreme of hyperfine splitting of the spectrum of the Mg^{2+}-containing proteoliposomes is wider than that of Mg^{2+}-free proteoliposomes, i.e., the order parameter of the former is higher than that of the latter. This suggests a more rigid environment

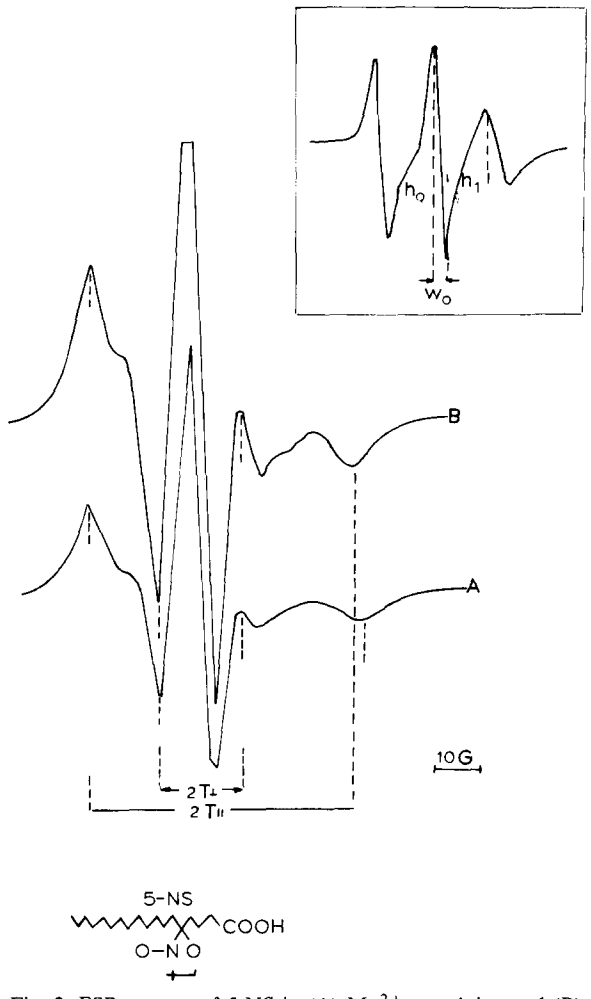

Fig. 2. ESR spectra of 5-NS in (A) Mg^{2+}-containing and (B) Mg^{2+}-free proteoliposome preparations. 50 μl redistilled diethyl ether were added to 2–3 μl of 0.25 mM 5-NS/$CHCl_3$ solution, dried under vacuum and 10 μl of 50 mM $K_3Fe(CN)_6$, proteoliposomes corresponding to 750 μg phospholipid and 10 mM Tris-H_2SO_4 (pH 8.0) were successively added so as to make up a total volume of 0.1 ml. Measurements were carried out at room temperature.

around the 5-NS spin label probe in the Mg^{2+}-containing proteoliposomes, which is located near the bilayer surface. This is consistent with the results obtained with the fluorescent probe anilinonaphthalenesulfonate, which is also located near the lipid/water interface.

The spin label 16-NS was employed to monitor the fluidity of the inner core of phospholipid bilayer, and spin label 12-NS for the zone intermediate to those monitored by 5-NS and 16-NS.

TABLE II

COMPARISON OF ESR PARAMETERS FOR THE SPIN LABELS 5-NS AND 16-NS IN THE TWO PROTEOLIPOSOME PREPARATIONS

The experimental conditions were the same as those in Fig. 2. See Materials and Methods for calculation of the order parameters (S) or rotational correlation time (τ_c). Values of S and τ_c are the mean ± S.E. with the number of experiments in parentheses. n.s., not significant.

Proteoliposome preparation	5-NS			16-NS
	$2T_{\parallel}$ (G)	$2T_{\perp}$ (G)	S	τ_c (s) ($\times 10^{-10}$)
$-Mg^{2+}$	57.8	19.4	0.676 ± 0.021(4)	21.9 ± 2.0(7)
$+Mg^{2+}$	58.8	18.4	0.723 ± 0.016(4)	22.8 ± 0.79(7)
			$P < 0.01$	n.s.

The 16-NS label is located in an almost non-ordered, isotropic environment, and thus its rotational correlation time can be calculated from the linewidth of the medium-field line (W_0) and the heights of the medium- and high-field lines (h_0, h_{-1}; see inset to Fig. 2). It is interesting that there was no significant difference between the τ_c values of the two proteoliposome preparations (Table II). Similar results were obtained with the 12-NS label. This means that the fluidity of the two proteoliposome preparations was not obviously different in the intermediate and deeper regions of the phospholipid bilayer.

CD study of the effect of Mg^{2+} on the conformation of reconstituted H^+-ATPase

Conformational changes in membrane proteins incorporated in phospholipid liposomes can be studied by CD spectroscopy [12]. We have, therefore, applied this technique to monitor the effect of Mg^{2+} on the conformation of the reconstituted H^+-ATPase.

Fig. 3 shows CD spectra in the wavelength range from 200 to 250 nm of H^+-ATPase reconstituted in the presence and absence of Mg^{2+}. Both spectra are characterized by two negative maxima at 208 and 219 nm. The CD spectrum of the Mg^{2+}-containing proteoliposomes has a larger negative ellipticity value than that of the Mg^{2+}-free proteoliposomes. The α-helix contents of the enzyme in the two preparations, estimated according to the 'three-wavelength' method [13] using the ellipticity values at 210, 219 and 225 nm, are 24% for the Mg^{2+}-containing H^+-ATPase preparation and 19% for the Mg^{2+}-free preparation. This means that reconstitution of the enzyme in the presence of Mg^{2+} leads to a 26% higher α-helix content.

The CD specturm might be distorted by such effects as light scattering, absorption dampening and absorption flattening, arising from the non-transparent particulate nature of biological membrane samples. Therefore, we have also recorded the CD spectra of liposomes without incorporated H^+-ATPase under the same conditions. In these

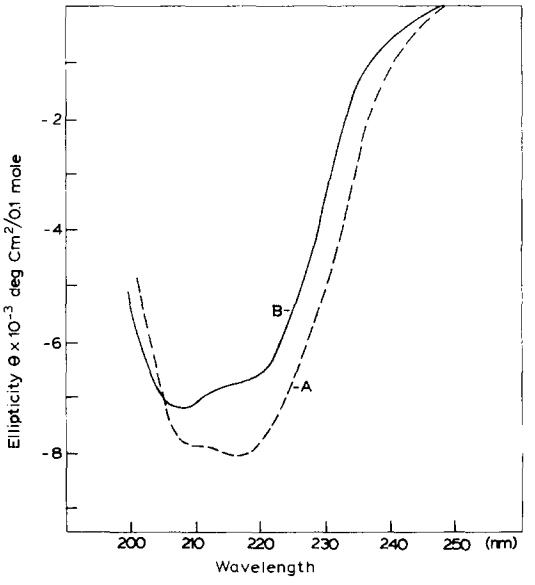

Fig. 3. CD spectra of the reconstituted enzyme. (A) Mg^{2+}-containing proteoliposomes and (B) Mg^{2+}-free proteoliposomes. Assay medium consisted of 0.15 ml solution containing 100 μg enzyme protein, 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.3 mM (dithiothreitol, 10% methanol and 0.1 mM Na_2ATP.

cases, neither positive nor negative peaks were detected in the CD spectrum in the 200–250 nm range. Later addition of Mg^{2+} to the Mg^{2+}-free preparation did not change either the absorption or the peak shift of the CD spectrum. This indicates that the changed CD spectrum of the Mg^{2+}-containing proteoliposome preparation is not due to the higher Mg^{2+} concentration as such, but rather to the induction of a conformational change in the H^+-ATPase when reconstituted in the presence of Mg^{2+}.

Discussion

Razin [14] reported that Mg^{2+} facilitated the reconstitution of the mycoplasma membrane. Kagawa [15] suggested that Mg^{2+} was essential for the phosphorylation activity of the reconstituted bacterial photosynthetic membranes, but was not necessary in the case of mitochondrial membrane reconstitution. Abrams and Baron [16] also reported that Mg^{2+} was required for reconstitution of the ATPase from *Streptococcus faecalis* with the plasma membrane. Our present results show that Mg^{2+} present during reconstitution of pig heart mitochondrial H^+-ATPase by the cholate dialysis method markedly and reproducibly increases the activity of the enzyme. The effects of other divalent cations on the H^+-ATPase reconstitution were also compared [6]. The effectiveness of enhancement of $^{32}P_i$-ATP exchange activity decreased in the order: $Mg^{2+} > Ca^{2+} > Mn^{2+} > Sr^{2+}$. When ranked in order of increasing the H^+-ATPase activity and its sensitivity to oligomycin, the following series emerged: $Ca^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+}$. Other bivalent metallic ions, e.g., Cd^{2+} or Zn^{2+}, had little or no effect. It was postulated that Mg^{2+} acts by neutralizing negatively charged groups on membrane proteins and lipids which interfere with membrane reconstitution by electrostatic repulsion [17]. However, our results show that the effect of Mg^{2+} on the H^+-ATPase reconstitution with soybean phospholipid liposomes was rather specific and cannot be solely explained by its carrying positive charges.

What then is the role of Mg^{2+}? The effect of Mg^{2+} on the reconstitution of the H^+-ATPase-containing liposomes might be complicated by several factors, such as changes in vesicle size [5] and detachment of the F_1 portion during the dialysis procedure. We have found that the H^+-ATPase exhibits a much higher affinity for the larger liposomes and 1 mM Mg^{2+} has no effect on the size of liposomes (unpublished results). It was also noticed that when F_1 was added after the dialysis procedure, the presence of Mg^{2+} in the dialysis medium had a marked effect on ATPase activity of the reconstituted enzyme and its sensitivity to oligomycin [6]. Hence, Mg^{2+} might play a certain role in the interaction between lipids and membrane proteins, or it might indirectly affect the physical state of the lipid bilayer. Therefore, we have compared the fluidity of the proteoliposome, reconstituted in the presence and absence of Mg^{2+}, by means of a fluorescent probe or ESR spin labels. Our results show a difference in ordering located near the lipid/water interface of the proteoliposomes. Those reconstituted in the presence of Mg^{2+} were less fluid than those reconstituted in its absence. The H^+-ATPase complex is known to consist of three components: the hydrophobic protein (F_0) which is buried in the lipid core, the soluble ATPase (F_1) and the oligomycin sensitivity-conferring protein connecting F_1 and F_0. The F_0 protein showed a marked increase in its α-helix content when incorporated into a lipid bilayer [18]. It was postulated that the conformation of F_0 buried in the hydrophobic portion depends on the physical state of the surrounding phospholipid molecules. The conformation of the soluble ATPase (F_1) will be influenced in turn by the conformational change of F_0 [19]. Therefore, we tentatively suggest that Mg^{2+} might play a role in altering the lipid packing near the surface of the bilayers, which would induce a suitable conformation of the reconstituted H^+-ATPase with higher enzymic activity. This assumption is supported by CD studies of the effect of Mg^{2+} on the conformation of the reconstituted H^+-ATPase, which indicate that the α-helical content of the enzyme in the Mg^{2+}-containing proteoliposomes is higher than that in the Mg^{2+}-free proteoliposomes. It is interesting to notice that the effect of Mg^{2+} on the incorporation of the H^+-ATPase complex depends on the method of reconstitution. It was more obvious in cholate dialysis than in the incubation method. This may be due to the simultaneous Mg^{2+} effect on both sides of the lipid bilayers in cholate dialy-

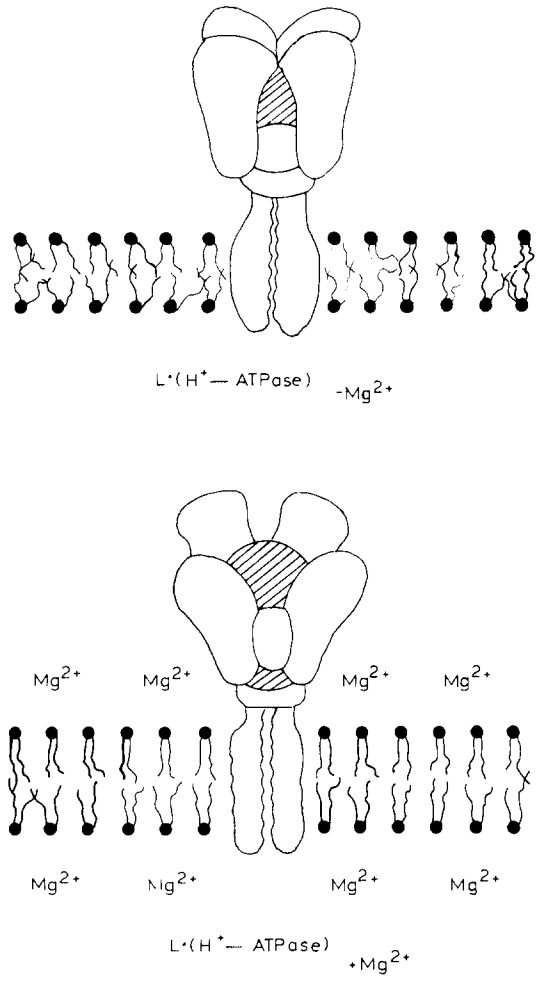

Fig. 4. Hypothetical scheme of the effect of Mg^{2+} on the reconstituting H^+-ATPase.

sis. Presumably, this may offer a proper physical state of the phospholipids in the bilayer, favoring the formation of a suitable conformation of the H^+-ATPase.

The role of Mg^{2+} in the reconstitution of the H^+-ATPase in liposomes may tentatively be summarized with the diagrams shown in Fig. 4. It may be added that Mg^{2+} also induces structural changes in the mitochondrial inner membrane and concomitant changes in its functional properties [20]. It is generally estimated that 70–80% of membrane proteins are intrinsic proteins, which are partially buried in the hydrophobic portion and partially in contact with the aqueous phase. Hence, the structure and function of many membrane proteins may be modulated by metal ions in a similar way.

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