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Further study on the role of Mg^{2+} in lipid–protein interaction in reconstituted porcine heart mitochondrial H^+-ATPase

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Porcine heart mitochondrial H^+-ATPase was reconstituted by cholate dialysis method in liposomes containing neutral (PC, PE), acidic (PG, PI, PA, PS, DPG) or neutral and acidic phospholipids. The Mg^{2+} effect on the ATPase activity and its sensitivity to oligomycin, ATP-induced $\Delta\Psi$ and ΔpH formation was observed for the proteoliposomes containing acidic but not neutral phospholipids. Maleimide spin labels with varying arm lengths or bromoacetamide spin probe were used to monitor the conformational difference of H^+-ATPase in the Mg^{2+}-containing and Mg^{2+}-'free' samples. A difference in W/S ratio (weakly immobilized/strongly immobilized component in the ESR spectra) could be detected for the $F_0 \cdot F_1$-containing and F_1-depleted, (F_0)-containing proteoliposomes, suggesting conformational difference in the F_0-F_1 complex and F_0 portion induced by the Mg^{2+} effect. A conformational change of the β-subunits in the F_1 portion was also deduced from the ATP-induced fluorescence quenching of aurovertin-complex for Mg^{2+}-containing samples. The results obtained are in favor of our previous assumption that Mg^{2+} may play its role by altering the physical state of the lipid bilayer, which would induce a conformational change in F_0 (buried in the lipid core), which in turn is transmitted to the catalytic F_1, resulting in a higher enzyme activity.

Introduction

It has been reported since 1974 that Mg^{2+} may play some role in the reconstitution of biological membranes and insertion of membrane proteins into artificial membranes. Rasin [1] suggested that Mg^{2+} facilitates the reconstitution of the mycoplasma membrane solubilized by sodium dodecyl sulfate. Kagawa [2] reported that Mg^{2+} was essential for the phosphorylation activity of the reconstituted bacterial photosynthetic membranes. Abrams and Baron [3] also found that Mg^{2+} ws required for reconstitution of the ATPase from *Streptococcus foccalis* with the plasma membrane. Sone et al. [4] suggested that Mg^{2+} promoted the reconstitution of the H^+-ATPase complex ($TF_0 \cdot F_1$) from the thermophilic bacterium PS3 in lipid vesicles, where both deoxylcholate and cholate were used to solubilize the membrane preparation. It has also been reported by Ernster and co-workers [5] that Mg^{2+} could improve the reconstitution efficiency of F_1 with F_0 in bovine heart mitochondrial H^+-ATPase complex. However, the mechanism of Mg^{2+} effect has not been studied in detail. In recent years, study on the reconstitution of porcine heart mitochondrial H^+-ATPase into soybean phospholipid vesicles by cholate dialysis has been carried out in our laboratory. We found that 1 mM Mg^{2+} in the dialysis medium could greatly enhance the ATPase activity, $^{32}P_i$-ATP exchange, ATP-driven membrane potential and ΔpH formation, as well as the sensitivity to oligomycin and DCCD of the reconstituted enzyme [6–8]. The effect of Mg^{2+} on the lipid fluidity and conformation of the H^+-ATPase by CD technique has been measured [6]. Based on the results obtained, we tentatively suggested that Mg^{2+} effect may offer a proper physical state of phospholipids, favoring the formation of a suitable conformation of the H^+-ATPase complex with higher enzyme activity [6].

The above-mentioned assumption is further supported by our recent research results which will be presented in the following.

Abbreviations: DCCD, *N'*,*N*-dicyclohexylcarbodiimide; oxonol-VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; ACMA, 9-amino-6-chloro-2-methoxyacridine; 33–48P, 38–45P, protein fractions precipitated within the range of ammonium sulfate saturation indicated; DTT, dithiothreitol; CD, circular dichroism; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; DPG, diphosphatidylglycerol; MSL, maleimide spin label; BrSL, bromoacetamide spin label.

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Materials and Methods

Materials

Porcine heart mitochondrial H^+-ATPase was prepared by the method of Kagawa and Racker as described in detail in a previous paper [9].

Soybean phospholipid was purchased from Beijing Nanyuan plant oil factory and purified as described in Ref. 9.

Phosphatidylcholine(PC), phosphatidylethanolamine (PE), phosphatidylglycerol(PG), phosphatidylinositol (PI), phosphatidylserine(PS), phosphatidic acid(PA) and diphosphatidylglycerol(DPG) were purchased from Sigma. Oxonol-VI and all the spin labels used were from Molecular Probes, Inc. ACMA from Dr. Y.G. Huang in our own institute. Trypsin and trypsin inhibitor were from Sigma. Aurovertin was a generous gift of Drs. P.V. Vignais and M. Satre from Grenoble, France.

All the other reagents used were of research grade. All-glass distilled water was used throughout the experiments.

Methods

Reconstitution. The H^+-ATPase-containing proteoliposomes were reconstituted as follows: 20 mg of phospholipid was dried under vacuum at room temperature and was dissolved in 0.08 ml of 8% sodium cholate (pH 8.0) and then 0.2 ml of the solution containing 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA and 0.64 mM dithiothreitol was added. The resulting suspension was subjected to sonic oscillation on ice in an MSE ultrasonic disintegrator to clarity. To the sonicated suspension 4 mg protein of the H^+-ATPase was added and divided into two portions, which were dialyzed against 300 volumes of the solution containing 10% methanol, 5 mM mercaptoethanol, 0.2 mM EDTA, 0.1 mM Na_2ATP, 10 mM Tris-HCl (pH 7.5) in the presence or absence of 1 mM Mg^{2+} for 22 h at 0–4 °C.

ATPase activity determination. The ATPase activity and its sensitivity to oligomycin and DCCD were assayed according to the method of Serrano et al. [10].

Protein was determined according to the method of Lowry et al. [11] using bovine serum albumin as standard.

Measurement of ATP-driven membrane potential and ΔpH formation. ATP-driven membrane potential was monitored by the absorbance change of the voltage-sensitive probe oxonol-VI [12]. The difference absorbance $\Delta A_{590-630\,nm}$ was recorded in a Hitachi 557 double-beam dual-wavelength spectrophotometer. ATP-driven ΔpH formation was monitored by following the fluorescence quenching of ACMA [13], which was recorded in a Hitachi 650-60 fluorescence spectrophotometer.

300 μg protein of the reconstituted H^+-ATPase-containing proteoliposomes was suspended in 2 ml of the medium containing 40 mM Tris-acetate (pH 7.5), 250 mM sucrose, 2 mM $MgSO_4$ and 4 μM oxonol-VI (for monitoring the membrane potential) or the same amount of the proteoliposomes was suspended in 2 ml of the medium containing 30 mM Tricine, 10 mM Mes (pH 7.5), 10% glycerol, 1 mM DTT, 50 mM KCl, 2 mM $MgSO_4$ and 50 μM ACMA (for monitoring the ΔpH formation). For both cases, the proton gradient was generated by adding 20 μl of ATP/$MgSO_5$ (1 : 1, 100 mM, pH 7.5). Changes in $\Delta A_{590-630\,nm}$ of oxonol-VI or ACMA fluorescence (excitation at 410 nm and emission at 475 nm) were followed as a function of time. The proton gradient was discharged with 5 μl of oligomycin (1 mg/ml in ethanol). The temperature was kept at 30 °C.

Spin labelling. To 5 mg of the H^+-ATPase in 0.14 ml of the solution containing 5 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.32 mM DTT, 0.5 M sucrose, 0.14 ml of the buffer containing 10 mM Tris-HCl (pH 8.0), 0.5 M sucrose (TS buffer) was added. Then, 0.03 ml of the 10 mM maleimide spin labels (MSL, in ethanol) or bromoacetamide spin label (BrSL, also in ethanol) was added. After gentle mixing, the mixture was kept at 8–10 °C for 16 h for the MSL labelling and for 72 h for the BrSL labelling. To the mixture, 22.5 ml TS buffer and 22.5 ml saturated ammonium sulfate were added on ice. After stirring for 5 min it was spun down at 20 000 × *g* for 20 min. The pellet was washed once again as above. The final pellet was dissolved in 0.1 ml TS buffer and protein determined.

Trypsin treatment. According to the method of Racker [14] to 0.4 ml of the MSL-I-labelled H^+-ATPase-containing proteoliposomes, 0.5 ml of the solution containing 10 mM Tris-HCl (pH 7.4), 0.2 mM EDTA was added and centrifuged at 105 000 × *g* for 20 min. The pellet was dissolved in 0.2 ml of the above solution and 0.1 ml of trypsin (6 mg/ml) was added. After incubation at 30 °C for 35 min, 1 ml trypsin inhibitor (0.6 mg/ml) and 0.4 ml of 8 M urea was added and kept on ice for 60 min. The suspension was centrifuged at 105 000 × *g* for 20 min. The pellet was suspended in 6.1 ml of 0.2 M sucrose and spun down again as above. The final pellet was dissolved in 0.1 ml of the proper dialysis buffer for ESR measurement.

ESR measurement. The ESR spectra were recorded in a Varian-109 spectrometer. The samples were introduced in the quartz capillary with diameter of about 1 mm, the protein concentration in the samples was about 10 mg/ml. Instrument settings were: central magnetic field, 3250 G; scan range, 250 G; microwave power, 5 mW; for other settings, see the legends.

Aurovertin fluorescence assay. Crystalline aurovertin was dissolved in methanol and the concentration determined from the absorbance at 367.5 nm using an extinction coefficient of 42 700 $M^{-1} \cdot cm^{-1}$ [15]. Fluorescence measurements were made in a Hitachi 650-60 fluorescence spectrophotometer. For aurovertin experi-

ments excitation wavelength of 370 nm and emission wavelength of 470 nm were used. All experiments were carried out in a solution composed of 10 mM Tris-H_2SO_4 (pH 7.7), 0.5 mM EDTA, 0.5 mM DTT, 50 mM sucrose at 30 °C [16]. 0.5 mg protein of the proteoliposomes was added to the cuvette containing the medium. The final volume was 2 ml. The fluorescence due to the lipid scattering was recorded and subtracted. Aurovertin was added to the cuvette to give a final concentration of 2 μM. The fluorescence was recorded and time scan at given excitation and emission wavelengths was performed. 15 μl of 200 mM ATP (pH 7.7) were injected into the cuvette while continuously monitoring fluorescence. The maximal ATP-induced decrease in aurovertin fluorescence was expressed as the percentage decrease following addition of ATP.

Results

Dependence of the Mg^{2+} effect on the nature of phospholipids

We have previously reported [6–8] that 1 mM Mg^{2+} during reconstitution could greatly enhance the ATPase activity, $^{32}P_i$-ATP exchange, ATP-driven membrane potential and ΔpH formation as well as its sensitivity to oligomycin or DCCD in the reconstituted H^+-ATPase complex. It seems that the Mg^{2+} effect might be interpreted as resulting mainly from the following: (a) Mg^{2+}-mediated change of the physical state of lipids in turn ensuring conformation of H^+-ATPase possessing higher activity, (b) direct interaction of Mg^{2+} with the H^+-ATPase. In an attempt to discriminate between these two possibilities, porcine heart mitochondrial H^+-ATPase was reconstituted in neutral (PC, PE), or acidic (PI, PG, PA, PS, DPG) phospholipid (instead of soybean phospholipid) liposomes separately. The ATPase activity as well as its sensitivity to oligomycin of the reconstituted enzyme were measured and compared. As shown in Fig. 1, 1 mM Mg^{2+} in the dialysis medium consistently (but unevenly) enhanced the ATPase activity and its sensitivity to oligomycin in the acidic phospholipid proteoliposomes, but had little or no effect in the case of neutral phospholipid proteoliposomes. Also, it can be seen in Fig. 1 that the Mg^{2+} effect on the reconstitution of soybean phospholipid proteoliposomes was quite similar to that of the various acidic phospholipid proteoliposomes. It was reported [17] that soybean phospholipid contains more than 40% of acidic phospholipids (mainly PI, PG and PA). Thus, the Mg^{2+} effect on the H^+-ATPase activity incorporated in soybean phospholipid vesicles might be a consequence of the interaction of Mg^{2+} with acidic constituents in soybean phospholipid.

It has been reported [18] that some acidic phospholipids alone might not easily form well-sealed liposomes by the cholate dialysis method, thus mixtures of neutral and acidic phospholipids were also examined. The effect of Mg^{2+} on the activity of PC + PE, or PC + PE + PG or PC + PE + DPG was compared in Table I which also showed that 1 mM Mg^{2+} in the dialysis medium had almost no effect on the ATPase activity as well as its sensitivity to oligomycin of the H^+-ATPase reconstituted in the PC + PE liposomes. The enhancing effect of Mg^{2+} appeared when PG or DPG was added together with PC + PE to reconstitute the H^+-ATPase.

As a proton translocator, the reconstituted mitochondrial H^+-ATPase can pump protons from outside to the interior of the vesicles using the energy released from hydrolysis of externally added ATP. Thus, a $\Delta\tilde{\mu}_{H^+}$ composed of transmembrane potential ($\Delta\Psi$) and trans-

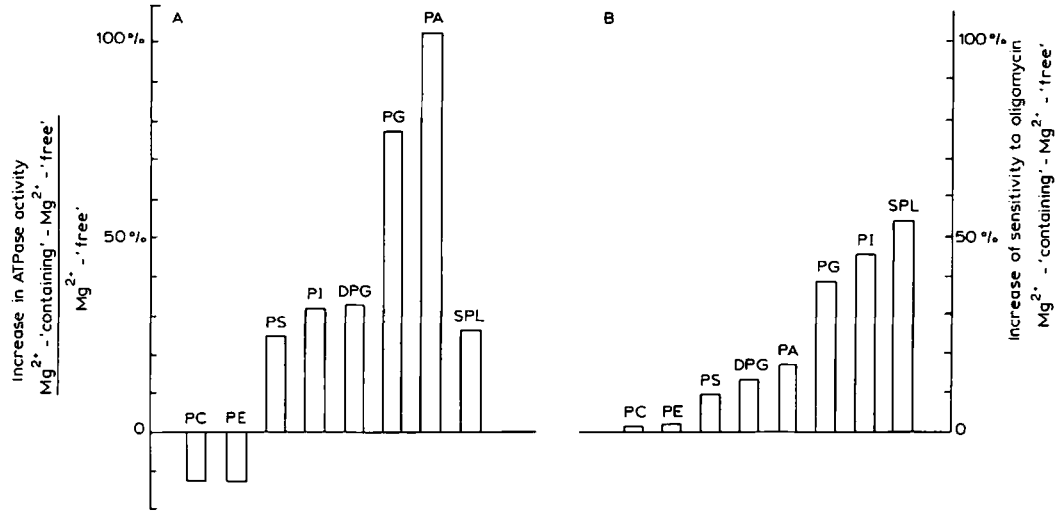


Fig. 1. Mg^{2+} effect on the ATPase activity (A) and its sensitivity to oligomycin (B) of the H^+-ATPase reconstituted in neutral or acidic phospholipid vesicles. The ATPase activity was measured at 30 °C, for 7 min, in the medium containing about 30 μg enzyme protein, 20 mM Tris-H_2SO_4 (pH 8.0), 0.5 mM EDTA, 2 mM $MgSO_4$, 20 μg pyruvate kinase, 1 μmol phospho*enol*pyruvate and 2.5 μmol Na_2ATP. To the medium 0.4 μg oligomycin was added for the inhibition assay. The enzyme activity is expressed as μmol P_i per mg protein per 7 min.

TABLE I

Mg^{2+} effect on the activity of the H^+-ATPase reconstituted on the PC + PE or PC + PE + PG or PC + PE + DPG vesicles

For experimental conditions, see the legend to Fig. 1.

Proteoliposomes	ATPase activity			Sensitivity to oligomycin (%)		
	$-Mg^{2+}$	$+Mg^{2+}$	increase (%)	$-Mg^{2+}$	$+Mg^{2+}$	increase (%)
PC/PE/PG						
1:1:0	9.77	8.96	−8.3	46.6	49.3	2.7
1:1:2	11.63	13.66	17.5	58.0	73.5	15.5
1:1:6	7.58	11.64	53.6	13.5	74.0	60.5
PC/PE/DPG						
1:1:0	10.63	9.27	−12.8	46.9	47.5	0.6
1:1:0.25	10.91	11.5	5.4	73.9	75.7	1.8
1:1:1	12.30	13.60	10.6	59.6	66.3	6.7
1:1:4	10.42	12.88	23.6	32.0	53.4	21.4

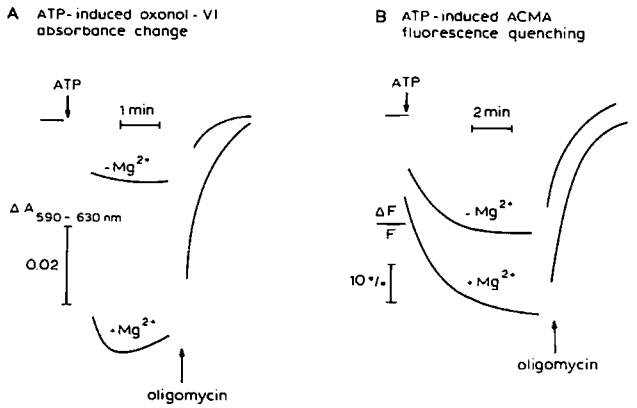


Fig. 2. Mg^{2+} effect on the ATP-dependent changes in oxonol-VI absorbance or ACMA fluorescence in H^+-ATPase-containing vesicles reconstituted with soybean phospholipid. For A, 300 μg protein of the H^+-ATPase-incorporating proteoliposomes was in 2 ml of the medium containing 40 mM Tris-acetate (pH 7.5), 250 mM $MgSO_4$ and 4 μM oxonol-VI. For B, the same amount of the proteoliposomes was in 2 ml of the medium containing 30 mM Tricine, 10 mM Mes (pH 7.5), 10% glycerol, 1 mM DTT, 50 mM KCl, 2 mM $MgSO_4$ and 50 μM ACMA. For both A and B, the proton gradient was generated by adding 20 μl of ATP/$MgSO_4$ (1:1, 100 mM, pH 7.5). Changes in $A_{590-630nm}$ of oxonol-VI or ACMA fluorescence (excitation at 410 nm and emission at 475 nm) were followed as a function of time. The proton gradient was discharged with ethanolic oligomycin (5 μl of 1 mg/ml), as shown by the arrows. The temperature was kept at 30 °C.

membrane pH difference (ΔpH) can be generated across the membrane. This pumping activity of the H^+-ATPase is a more significant measure of its function. Here, by using the voltage-sensitive probe oxonol-VI and the pH-sensitive probe ACMA, the ATP-driven $\Delta\Psi$ and ΔpH were measured and compared separately. From Fig. 2 it can be seen that for the H^+-ATPase-incorporating soybean phospholipid proteoliposomes, 1 mM Mg^{2+} in the dialysis medium markedly increased the maximal ATP-induced oxonol-VI absorbance change or the ACMA fluorescence quenching. The initial rates of oxonol-VI absorbance change or ACMA fluorescence quenching were also much higher for the Mg^{2+}-containing proteoliposomes. It was also noted that the Mg^{2+} effect could not be detected for the proteoliposomes reconstituted with only neutral phospholipid (PC + PE), but appeared when acidic phospholipid (PG) was present (Table II). This indicated that the Mg^{2+} effect on the proton pumping activity of the H^+-ATPase was also dependent on the presence of acidic phospholipids in the liposomes. It is also worthy of note that increase of the ATPase activity and its sensitivity to oligomycin of different acidic phospholipid proteoliposomes by Mg^{2+} varies greatly (Fig. 1). So these changes affected by Mg^{2+} may also dependent on the nature of the acyl chain of acidic phospholipids.

TABLE II

Mg^{2+} effect on the ATP-dependent changes in ACMA fluorescence or oxonol-XI absorbance in the H^+-ATPase-incorporating vesicles reconstituted with PC + PE or PC + PE + PG

For experimental conditions, see the legend to Fig. 2.

Proteoliposomes	Quenching in ACMA fluorescence				$\Delta A_{590-630nm}$ change in oxonol-VI absorbance			
	maximal quenching (%)		initial rate of quenching (%·min^{-1})		maximal change		initial rate of change (min^{-1})	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
PC/PE = 1:1	22.7	21.2	16	16	0.021	0.019	0.031	0.030
PC/PE/PG = 1:1:2	41.2	65.4	48	97	0.053	0.080	0.066	0.129

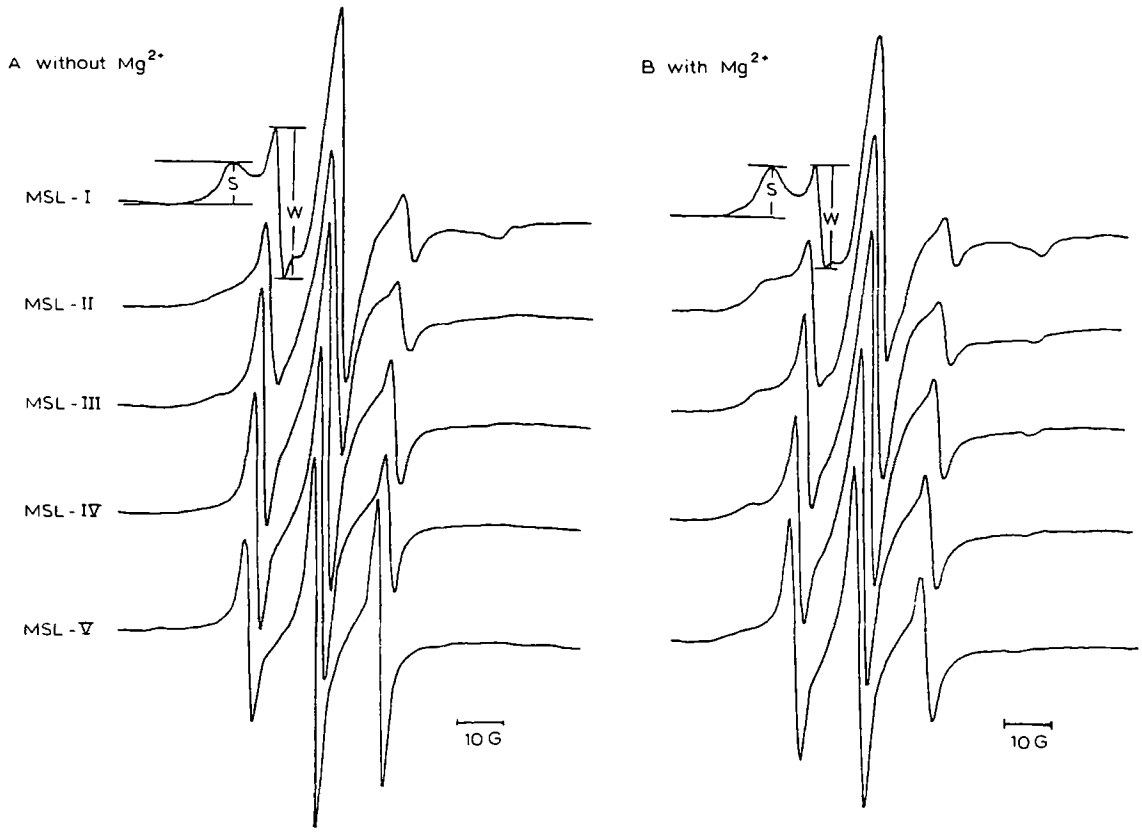


Fig. 3. ESR spectra of MSL(I)–(V) labelled L (H^+-ATPase) without Mg^{2+} and L (H^+-ATPase) with Mg^{2+}. Microwave power, 5 mW; modulation, 3.2 G; time constant, 1 s; scan time 8 min, 30 °C.

Study of the Mg^{2+} effect on the conformation of reconstituted H^+-ATPase using spin labels

In a previous paper [6] the conformation of H^+-ATPase in the Mg^{2+}-containing and Mg^{2+}-'free' proteoliposomes have been compared by circular dichroism (CD) spectra. In an attempt to further explore the difference in the molecular arrangement of H^+-ATPase in these two proteoliposomes, five thiolphilic maleimide and bromoacetamide spin probes were used. The structural formular of these probes are shown in Table III. It is well known that sulfhydryl groups are present in both F_0 and F_1 portions of mitochondrial H^+-ATPase [19–23]. Based on protein sequence analysis, Walker et al. [19] reported that there are eight cysteine residues in beef heart mitochondrial F_1, two in β subunit and one in each γ and ϵ subunit. But until now it is not known how many sulfhydryl groups are present in the F_0 portion.

For labelling, excess amount of each spin probe was used to react with the porcine heart mitochondrial H^+-ATPase complex. The unreacted labels were washed away by repeat precipitation with ammonium sulfate and centrifugation. The supernatant following each centrifugation was examined by ESR measurement until all the unreacted labels have been completely removed. If the enzyme complex was pretreated with *N*-ethylmaleimide (a sulfhydryl modifier), no spin labels could be bound to the enzyme complex. This indicate that the sulfhydryl groups in the enzyme complex could be covalently modified by the MSL spin labels and used for monotoring conformational change.

The labelled H^+-ATPase was then reconstituted in soybean phospholipid liposomes by cholate dialysis in the presence or absence of 1 mM $MgCl_2$. The ESR spectra were recorded and compared. Fig. 3 shows the spectra of maleimide probes-labelled proteoliposomes. The distance between the reacting double bond of the maleimide group and the free radical on the N-O bound of MSL-I-MSL-V increases from 6.8 to 15.3 Å. In the case of MSL-I, the spectrum is a composite of at least two components, one due to spin labels which have no independent motion relative to the enzyme molecule to which they are bound and another due to spin labels which have some independent motion. The heights of the two components in the spectra are designated as S and W (strongly immobilized component and weakly immobilized component). The ratio W/S has been taken as a conformational index of membrane proteins by many authors [24–26]. With increase in the arm length of MSL labels, the strongly immobilized component will become less prominent. This may be explained as such that for the label with longer arm, it projects out more

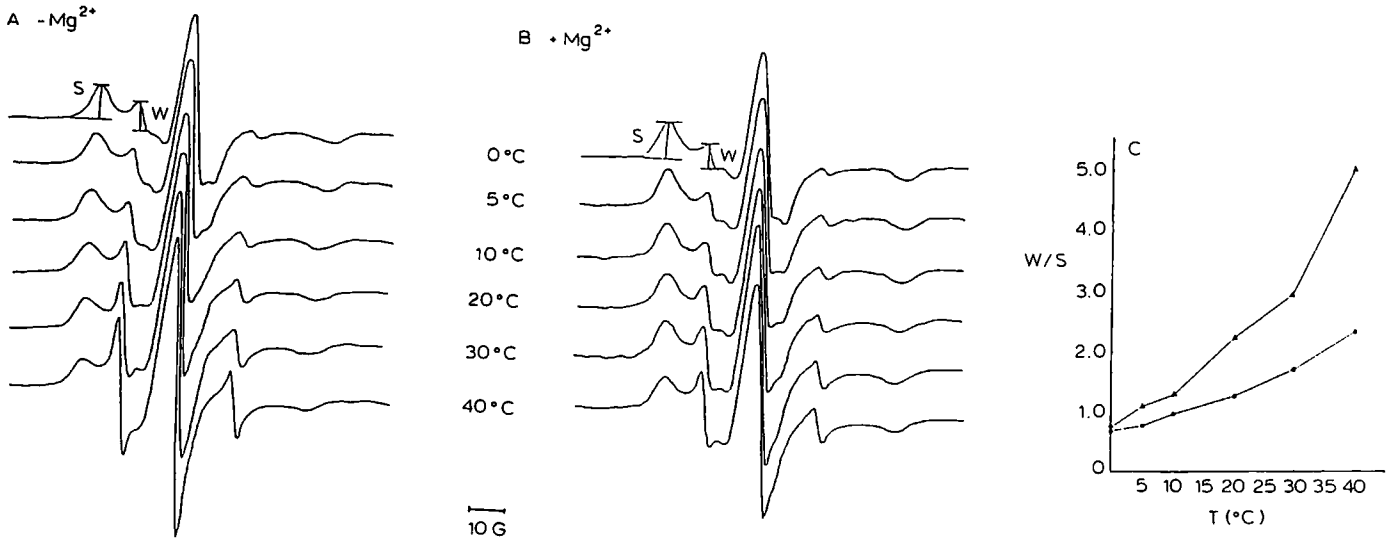


Fig. 4. ESR spectra of MSL(I)-labelled L·(H^+-ATPase) without Mg^{2+} (A) and L·(H^+-ATPase) with Mg^{2+} (B) recorded at different temperatures. Microwave power, 5 mW; modulation, 3.2 G; time constant, 0.5 s; scan time, 4 min. (c) Plot of W/S against temperatures.

from the site to which it is bound, so there is less restriction from the enzyme molecule on the independent motion of the proxyl ring of the label.

When the spectra on the left side of Fig. 3 are compared with those on the right side, it can be clearly seen that for MSL-I labelled, the W/S ratio between the Mg^{2+}-'free' and the Mg^{2+}-containing proteoliposomes still exist, but with increasing in arm length of label compounds, the difference become less and less obvious. So, it indicates that the labels with shorter arm are more sensitive for the monitoring of conformational change in the incorporated H^+-ATPase to which it is bound. Hence, the MSL-I was used as probe in the following experiments.

The ESR spectra of MSL-I labelled H^+-ATPase-incorporating proteoliposomes reconstituted in the presence or absence of Mg^{2+} were also recorded at various temperatures (Fig. 4). The W/S ratio was calculated and plotted against temperature (Fig. 4C). It could be seen that the W/S is consistantly lower for the Mg^{2+}-containing proteoliposomes than that of Mg^{2+}-'free' ones.

Marsh has suggested [27] that for conformational study, iodoacetamide spin labels are more sensitive to conformational changes due to their more flexible arm and this was verified in conformational study of the sarcoplasmic reticulum Ca^{2+}-ATPase [28,29]. Here, an analog, bromoacetamide spin label (BrSL) was also used for measuring the conformational difference between Mg^{2+}-'free' and Mg^{2+}-containing samples. From Fig. 5 it can also be noticed that a larger W/S ratio was obtained in the former case.

Summing up, the results obtained using MSL derivatives and bromoacetamide as spin probes provide further evidence that a difference in conformation of H^+-ATPase may exist between the Mg^{2+}-containing and Mg^{2+}-'free' proteoliposomes. Since the strongly immobilized component from the spectra was thought to be due to the labels bound to deeply buried sulfhydryl groups and the weakly immobilized component originate from less deeply buried ones, it may be deduced from the ESR spectra that more sulfhydryl groups in the H^+-ATPase molecule become deeply buried in Mg^{2+}-containing proteoliposomes.

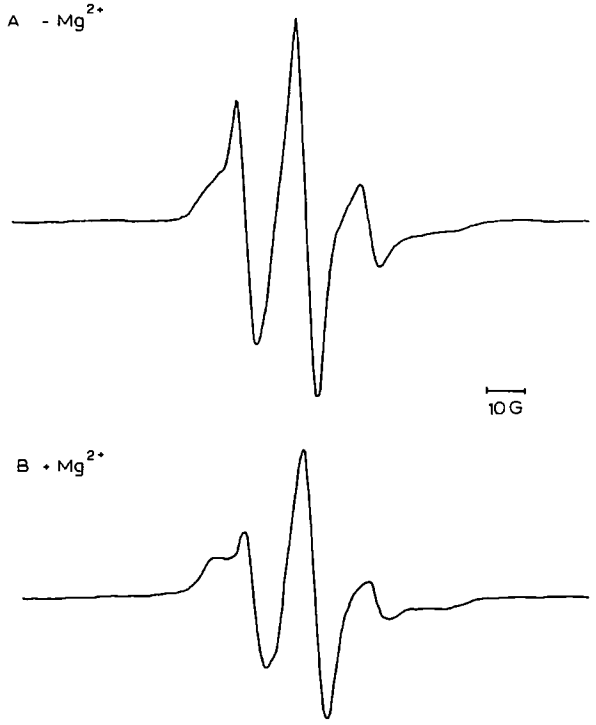
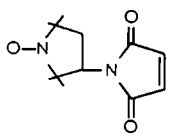
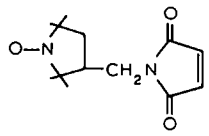
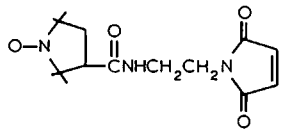
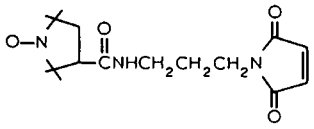
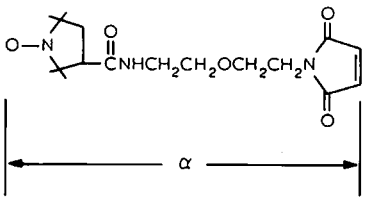
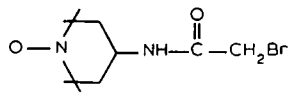


Fig. 5. ESR spectra of BrSL labelled L·(H^+-ATPase) without Mg^{2+} (A) and L·(H^+-ATPase) with Mg^{2+} (B). Microwave power, 5 mW; modulation, 2.5 G; time constant, 0.5 s; scan time, 4 min, 20 °C.

TABLE III

The structural formulae of the spin labels used

Labels	Structure	*d* (Å)
MSL		
I		6.8
II		7.9
III		11.6
IV		12.9
V		15.3
BrSl		

Conformational difference of F_1-depleted H^+-ATPase complex (F_0) between Mg^{2+}-containing and Mg^{2+}-'free' proteoliposomes

If the Mg^{2+} effect is indirect, the altering in lipid fluidity in the presence of Mg^{2+} during reconstitution would first induce a change in conformation of F_0 (buried in the lipid core) of H^+-ATPase and such change would then be transmitted to the soluble F_1 portion. In order to verify such assumption, conformational difference of F_1-depleted-H^+-ATPase (F_0) in Mg^{2+}-containing and Mg^{2+}-'free' proteoliposomes was studied by using MSL-I probe. First of all, the labelled H^+-ATPase was reconstituted in phospholipid liposomes in the presence or absence of Mg^{2+}. Then, both proteoliposomes were treated with trypsin and urea to remove the F_1 portion from the reconstituted H^+-ATPase so that no ATPase could be detected in the treated proteoliposomes as described in Ref. 14. Then, the ESR spectra of the remaining F_0 portion from Mg^{2+}-containing and Mg^{2+}-'free' samples were compared. Sulfhydryl groups are present both in F_0 and F_1 portions of H^+-ATPase. By spectrum integration it was found that the ESR signal from the depleted F_1 accounted for 2/3 of the total value, whereas the signal from remaining F_0 portion accounted for 1/3. The results show that the W/S ratio calculated from the ESR spectra of remaining F_0 portion was still lower in the Mg^{2+}-containing proteoliposomes than in the Mg^{2+}-'free' ones (Fig. 6). For the F_1 portion no comparison of ESR measurements could be made due to the hydrolysis of the enzyme protein by trypsin. So, in a separate experiments, the Mg^{2+} effect on the conformation of purified F_1 was investigated. The results clearly showed that no change in ESR spectra of MSL-I labelled F_1 could be

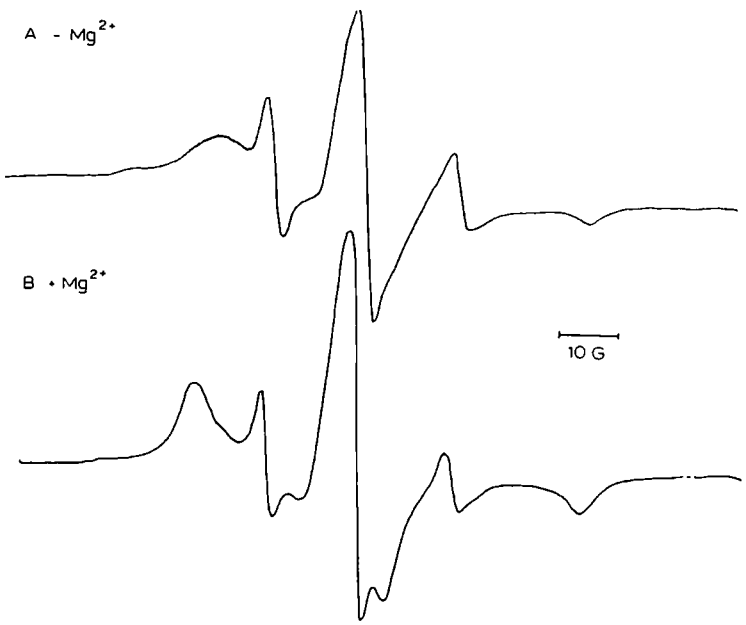


Fig. 6. ESR spectra of F_1-depleted L·(H^+-ATPase) without Mg^{2+} (A) and L·(H^+ATPase) with Mg^{2+} (B) labelled with MSL(I). Microwave power, 5 mW; modulation, 2.5 G; time constant, 0.5 s; scan time, 8 min, 18° C.

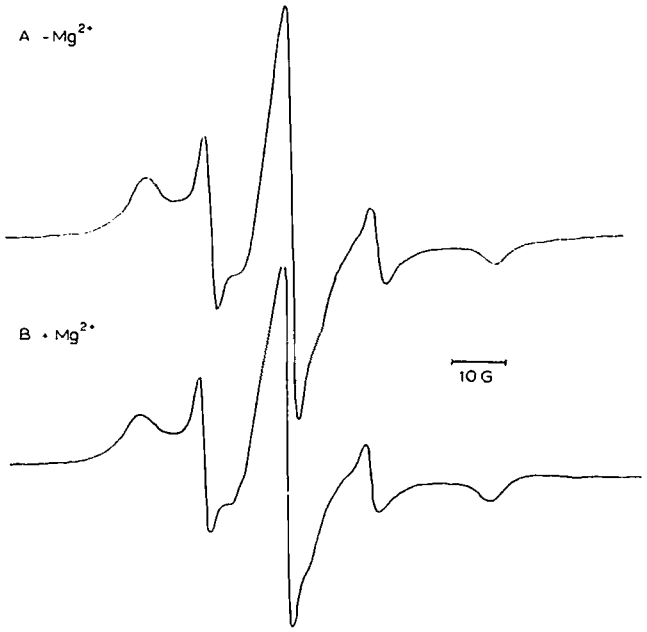


Fig. 7. ESR spectra of MSL(I)-labelled purified F_1 dialyzed in the presence (B) or absence (A) of 1 mM Mg^{2+} for 20 hours. F_1 was purified from porcine heart mitochondria. Triton X-100 was used to solubilize the mitochondria and F_1 was obtained and purified by step-wise ammonium sulfate precipitation. F_1 was labelled and washed by the same procedure as that for F_0-F_1 complex and then dialyzed for 20 h in the presence or absence of 1 mM $MgCl_2$ using the same dialysis medium for reconstitution of the $F_0 \cdot F_1$-containing proteoliposomes. Microwave power, 5 mW; modulation, 2.5 G; time constant, 0.5 s; scan time, 8 min, 18 °C.

detected following Mg^{2+} treatment by the same procedure as reconstitution experiments (Fig. 7).

Comparison of F_1 conformation of H^+-ATPase (F_0F_1) reconstituted in the presence or absence of Mg^{2+}

If the Mg^{2+} effect is not direct, the conformational change of F_0 caused by Mg^{2+}-mediated altering of the physical state of phospholipid would be transmitted to F_1. F_1 is composed of five subunits ($\alpha_3\beta_3\gamma\delta\epsilon$). It has been shown that aurovertin could be used as a probe for the conformational changes of β-subunit of F_1 from bovine heart or *Escherichia coli* [30–32]. Aurovertin exhibits very little fluorescence in either aqueous or non-polar solvents, but forms a highly fluorescent complex with F_1. It was demonstrated that a decrease in fluorescence intensity of aurovertin bound with free or membrane-associated F_1 could be observed following addition of saturating concentration of ATP. Such ATP-induced fluorescence quenching of the enzyme-aurovertin complex has been shown to be a consequence of the conformational change in β-subunits of H^+-ATPase complex [33–37].

For the aurovertin-labelling experiments, the (38–45 P) H^+-ATPase preparation (instead of 33–48 P) was used. Usually, the increase in fluorescence intensity of F_0F_1-aurovertin complex over that of free aurovertin

TABLE IV

Mg^{2+} effect on the ATP-induced $\Delta A_{590-630\,nm}$ changes in oxonol-VI absorbance and decreases in aurovertin fluorescence of the H^+-ATPase-incorporating vesicles reconstituted with PC OR PC + DPG

The ATP-induced $\Delta A_{590-630\,nm}$ changes in oxonol-VI absorbance were determined as described in the legend to Fig. 2. For aurovertin experiments, 0.5 mg protein of the proteoliposomes was added to the cuvette with 2 ml of medium containing 10 mM Tris-H_2SO_4 (pH 7.7), 0.5 mM EDTA, 0.5 mM DTT, 50 mM sucrose at 30 °C. Aurovertin was added to give a final concentration of 2 μM. 15 μl of 200 mM ATP (pH 7.7) were injected into the cuvette. The maximal ATP-induced decrease in aurovertin fluorescence (excitation at 370 nm and emission at 470 nm) was expressed as the percentage decrease following addition of ATP.

Proteoliposomes	ATP-induced $\Delta A_{590-630\,nm}$ changes in oxonol-VI absorbance		ATP-induced decrease in aurovertin fluorescence (%)	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
PC	0.005	0.004	21.2	19.3
PC/PG = 3:1	0.019	0.036	20.0	13.1

was about 17 times. It was also found that soybean phospholipid contains some unknown fluorescent substance with excitation and emission spectra overlapping with those of aurovertin. Several kinds of PE from different sources were also found to contain such unknown substance. So, only PC and DPG were used to reconstitute the enzyme. It can be seen in Table IV, when PC alone was used to reconstitute the enzyme, the ATP-driven changes in $\Delta A_{590-630\,nm}$ of oxonol-VI was rather small. This indicates that the pumping activity of the H^+-ATPase is rather low in this case. Also, almost no Mg^{2+} effect could be observed. But, when DPG was present, the pumping activity was increased and a strong Mg^{2+} effect appeared. In the case of PC reconstituted proteoliposomes, a slight difference in ATP-induced fluorescence quenching of aurovertin-H^+-ATPase complex exists between the Mg^{2+}-'free' and Mg^{2+}-containing samples. Perhaps, Mg^{2+} itself may also affect the fluorescence quenching of the aurovertin-enzyme complex. But, such difference became much more obvious in the case of PC + DPG proteoliposomes. So, from the result, it may be deduced that a difference in the conformation of β-subunit in the F_1 portion was involved in the Mg^{2+}-mediated effect on the reconstituted H^+-ATPase complex.

Discussion

The Mg^{2+} effect in enhancing the reconstituted H^+-ATPase activity might be interpreted as resulting from one of the following: (i) Mg^{2+}-mediated change of the physical state of lipids in turn ensuring conformation of H^+-ATPase possessing higher activity [5–8]; (ii) Mg^{2+}

could prevent detachment of F_1 from the H^+-ATPase complex during reconstitution by cholate dialysis [38]; and (iii) direct interaction of Mg^{2+} with the enzyme protein of H^+-ATPase. We have postulated that Mg^{2+} effect may be interpreted as resulting mainly from (i). This assumption is further supported by the experimental results presented here: (a) The Mg^{2+} effect on the reconstitution of H^+-ATPase was observed only with the acidic phospholipid but not with the neutral phospholipid proteoliposomes. (b) The ATPase complex is known to consist of three components: hydrophobic protein F_0, which is buried in the lipid core, the soluble ATPase F_1 and the stalk connecting F_0 and F_1. It is postulated that the conformation of F_0 depends on the physical state of the surrounding phospholipid molecules. The conformation of the bound F_1 will be influenced in turn by the conformation of F_0 [39]. So, if the Mg^{2+} effect on the reconstitution of H^+-ATPase is not a consequence of interaction of Mg^{2+} with the protein, the conformational change in the F_0 portion following Mg^{2+}-mediated altering in fluidity of phospholipid bilayer would be detected. The results obtained showed that using MSL-I as probe a difference in ESR spectra of the F_1-depleted H^+-ATPase (F_0) in Mg^{2+}-containing and Mg^{2+}-'free' proteoliposomes could be observed. No change in ESR spectra of MSL-labelled purified F_1, on the other hand, could be detected following Mg^{2+} treatment by the same procedure as in reconstitution experiments. A conformational change of β-subunits in the F_1 portion could also be detected from experiments of ATP-induced aurovertin fluorescence quenching of the H^+-ATPase-incorporating proteoliposomes reconstituted in the presence of Mg^{2+}.

Summing up, Mg^{2+} may play a role in altering the lipid fluidity of the bilayers, which would induce a change of conformation of F_0 portion of H^+-ATPase complex. Such a change could be transmitted to the soluble F_1 portion, the conformation of which is in turn altered, resulting in higher enzymic activity.

It has been reported that divalent cations can induce a serious of changes in the physical state of acidic phospholipid-containing bilayers, such as neutralization of the surface charge, increase of the surface pressure, enhancement of lipid phase transition temperature, decrease of the lipid fluidity in the bilayer [40–44]. There are several observations concerning the consequences that modifications in the physical state of lipids by divalent cations may have on the function of membrane proteins. Wojczak [45] found that the membrane surface potential can affect the activity of membrane enzymes and transport proteins. It was interpreted that lowering negative charge on the membranes by divalent cations could increase the activity of the enzyme using negatively charged molecules as substrate. They suggested that the neutralization of negative charge on the membrane by divalent cations may facilitate the binding of substrate to the enzyme. But Peng and Yang [46] from our laboratory has found that three was no difference in K_m between Mg^{2+}-'free' and Mg^{2+}-containing H^+-ATPase-incorporating vesicles, while the V_m was significantly higher in the later case. So, it seemed that the Mg^{2+} effect in our case could hardly be interpreted in terms of its facilitating binding of negatively charged ATP to the reconstituted H^+-ATPase.

Mg^{2+} is one of the most abundant cations within cells and its concentration is high in mitochondria, particularly within the matrix [47]. The free concentration of Mg^{2+} in the liver mitochondria has been estimated to be 1.6 mM [48]. Mg^{2+} could interact with the mitochondrial inner membrane [49] and induce structural changes [50]. It has also been reported that Mg^{2+} is necessary for the maintenance of membrane integrity [51]. But little attempt has been made to study the mechanism of such an effect.

We have also found that similar to the reconstitution of porcine heart mitochondrial H^+-ATPase into liposomes, Mg^{2+} may enhance the enzyme activity of reconstituted cytochrome *c* oxidase [52], porcine kidney medulla Na^+,K^+-ATPase [53], Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum and chloroplast H^+-ATPase (unpublished results). It is generally estimated that 70–80% of membrane proteins are intrinsic proteins, most of which are partially buried in the hydrophobic portion and partially in contact with the aqueous phase. Hence, the structure and function of many membrane enzymes and proteins may be regulated by Mg^{2+} in a similar way.

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