Effect of Lipid Autoperoxidation on the Activity of the Sarcoplasmic Reticulum (Ca$^{2+}$-Mg$^{2+}$)ATPase Reconstituted into Egg Yolk Phosphatidylcholine Bilayers

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Abstract. The activity of (Ca$^{2+}$-Mg$^{2+}$)ATPase reconstituted into egg yolk phosphatidylcholine liposomes is reduced when the lipid has been oxidized before the reconstitution procedure. It is suggested that the reversible decrease in activity is caused by a decrease of the lipid bilayer thickness due to the shortening of lipid acyl chains during autoperoxidation; the irreversible decrease in the activity is caused by chemical reactions of the enzyme SH groups with the lipid autoperoxidation products.

Key words: Sarcoplasmic reticulum (Ca$^{2+}$-Mg$^{2+}$)ATPase — Enzyme-lipid interaction — Lipid autoperoxidation — SH groups — Bilayer thickness

Introduction

The effect of lipid autoperoxidation on the activity of the (Ca$^{2+}$-Mg$^{2+}$)ATPase of sarcoplasmic reticulum (ATP phospho-hydrolase, E.C.3.6.1.3) has been known for more than 10 years (Hess et al. 1981; Kagan et al. 1983a,b; Klaan et al. 1983; Arkhipenko et al. 1983; Rubtsov et al. 1984). Induction of lipid autoperoxidation has been observed to result in an impairment of the calcium transporting function due to an increased membrane permeability for calcium ions (Kagan et al. 1983b); the degree of damage to the Ca$^{2+}$ transport system correlated with the concentration of lipid autoperoxidation products (Kagan et al. 1983a). Lipid autoperoxidation also increases the polarity and decreases the mobility of lipids in the sarcoplasmic reticulum membrane (Klaan et al. 1983), and reduces the number of DTNB accessible SH groups in the membrane protein (Kagan et al. 1983a; Rubtsov et al. 1984) which also might affect the enzyme activity. In the sarcoplasmic reticulum membranes the relative contributions of various effects of lipid autoperoxidation on ATPase activity are interconnected. To discriminate...
between the effects of the lipid bilayer itself and those of the possible lipid autoperoxidation products (protein chemical interactions), we studied the activity of pure (Ca^{2+}-Mg^{2+})ATPase reconstituted into liposomes prepared from oxidized phosphatidylcholine.

**Materials and Methods**

**Chemicals**

Potassium cholate was prepared by allowing potassium hydroxide act on cholic acid; the product was purified by diethyl ether extraction to give a white solid on rotary evaporation. n-Decane was purchased from Sigma. Egg yolk phosphatidylcholine (EYPC) was isolated from hen egg yolk as described by Singleton et al. (1965). Its purity was checked by two dimensional thin-layer chromatography. The obtained product was stored in chloroform:methanol solution (2:1) in a sealed bottle under nitrogen at −20 °C. Aliquots were taken, dried completely under a gentle flow of nitrogen and allowed to peroxidize freely in the laboratory. Peroxidation index (α) was measured repeatedly, and aliquots showing an appropriate index were stored in the same chloroform-methanol solution under conditions as above.

The intensity of peroxidation was measured as the absorbance ratio at 233 nm and at 215 nm (peroxidation index α) 3.25 × 10^{-4} mol/l lecithin in ethanol for UV spectroscopy (Klein 1970).

**Fatty acid determination**

One mg of EYPC was hydrolyzed in 2 ml 0.5 mol/l KOH solution in ethanol supplemented with a drop of benzene for 1 h at 80 °C under nitrogen gas. After recooling, 1 ml of water was added and fatty acids were extracted into 4 ml of hexane. The aqueous layer was removed, acidified with 0.5 ml of 4 mol/l HCl, and re-extracted three times with 4 ml of hexane. The collected hexane layers were evaporated to dryness and the solid was dissolved in 4 ml of 10% methanol in diethylether. Free fatty acids in this solution were esterified using reaction with diazomethane. After hydrolysis and esterification, fatty acid methyl esters were estimated using gas chromatography (HRGC 5160, Carlo Erba Instruments). Glass capillary columns (70 m) were filled with 19% SP 2340. Hydrogen was used as the carrying gas (Bohov et al. 1984). The composition of the fatty acid mixture was obtained in weight % and recalculated in molar % using the molecular weights of the particular fatty acid.

**Preparation of (Ca^{2+}-Mg^{2+})ATPase**

(Ca^{2+}-Mg^{2+})ATPase was prepared according to the method outlined by Warren et al. (1974) with slight modifications. A young female rabbit (New Zealand White), weighing about 2.5 kg, was killed and bled. All subsequent operations were carried out at 4 °C. White muscle was dissected from the hindlegs and the spinal region, finely chopped on a glass plate and excessive fat and connective tissues removed. A 100 g batch of white muscle was homogenized in a Waring blender for 30 s with 200 ml cold 0.3 mol/l sucrose, 20 mmol/l histidine, 1 mmol/l dithiothreitol (DTT) and 5 μmol/l phenylmethylsulfonyl fluoride (PMSF) (pH 8.0). The homogenate was centrifuged for 15 min (8000 × g, 4 °C). Undisrupted material was retreated as above. The pooled supernatants were filtered
through fine muslin and centrifuged for 90 min (37,000 × g 4°C). The pellets were resuspended in cold 0.3 mol/l sucrose, 10 mmol/l histidine, 0.6 mol/l KCl, 1 mmol/l DTT and 5 μmol/l PMSF (pH 8.0) (0.5 ml/g muscle) using a homogenizer tube fitted with a pestle. This homogenate was then centrifuged for 20 min (4000 × g, 4°C). The supernatant was then centrifuged for 90 min (37,000 × g, 4°C). The pellet was resuspended in 3–5 ml of 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0), transferred into dialysis tubing and dialysed overnight against 1 litre of the same buffer. To purify (Ca²⁺-Mg²⁺)ATPase, sarcoplasmic reticulum (SR) was solubilised by adding potassium cholate (100 mg/ml) in the same buffer, to give a final ratio of 0.4 mg cholate/mg protein. This material was then loaded onto a discontinuous sucrose gradients. Each gradient tube contained 1 ml of 60% w/v, 7 ml of 30% w/v and 5 ml of 20% w/v sucrose in cold 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0). The solubilised SR was layered onto the gradients to give 40–45 mg of protein/tube. The gradients were then centrifuged for 18 hours (95,000 × g, 4°C). The pure enzyme was collected from the 30%/60% interface and was washed by suspending in five volumes of 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0) and centrifuged for 1 hour (95,000 × g, 4°C). The pellet was resuspended in 3 ml of the same buffer, transferred into dialysis tubing and dialysed overnight against 1 litre of gently stirred 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0), containing 10 g of washed Amberlite XAD-2 ion-exchange resin to remove residual cholate. The final preparation was aliquoted and stored at −20°C. The total protein concentration was determined by measuring the absorbance at 280 nm (Hardwicke and Green 1974).

Reconstitution of (Ca²⁺-Mg²⁺)ATPase

The reconstitution of (Ca²⁺-Mg²⁺)ATPase into the phospholipid vesicles was done as follows: EYPC 0.5 mg (dried in a stream of nitrogen) was added to 25 μl buffer (0.25 mol/l sucrose, 1 mol/l KCl, 5 mmol/l MgATP, 50 mmol/l potassium phosphate pH 8.0) which contained 0.75 mg potassium cholate. The content was sealed under dry nitrogen, briefly vortex-mixed and sonicated to clarity (40 s in an 80 KHz sonicating water bath). (Ca²⁺-Mg²⁺)ATPase was added (0.64 μg) and the contents briefly vortex-mixed. The mixture was placed into a refrigerator for 1 h and subsequently diluted 10-fold with 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l potassium phosphate pH 8.0. The mixture was placed on ice for no longer than 2 h.

Determination of the (Ca²⁺-Mg²⁺)ATPase activity

ATPase activity was determined using a slight modification of the coupled assay system outlined by Warren et al. (1974). In brief, 50 μl of ATPase sample was added to 2.32 ml of buffer (40 mmol/l HEPES, pH 7.2) containing MgSO₄ (5.1 mmol/l), ATP (2.1 mmol/l), phosphoenolpyruvate (0.53 mmol/l), EGTA (1.02 mmol/l), NADH (0.152 mmol/l), 7.5 IU of pyruvate kinase (EC 2.7.1.40), and 18 IU of lactate dehydrogenase (EC 1.1.1.27). In the final assay medium the molar ratio of phospholipid to ATPase was approximately 1000:1. After incubation at 37°C for 15 min, the reaction was started by the addition of 90 μl CaCl₂ (25 mmol/l) and the decrease of absorbance at 340 nm was measured. Enzyme activity U (in units per mg enzyme) was calculated according to the equation:

\[ U/mg = (A_{340} \cdot V_{ass}) / (6.22 \cdot m_{prot}) \]

where \( A_{340} \) is the change in absorbance per min, \( V_{ass} \) is the final assay volume in ml, and \( m_{prot} \) is the weight of protein in the assay volume in mg. Measurements were made using
Filípek et al.

a DU-65 spectrophotometer (Beckman, USA). The activity of the (Ca$^{2+}$-Mg$^{2+}$)ATPase before the reconstitution varied between 11 and 12 U/mg and did not change during the long time (up to 8 months) storage at $-20^\circ$C. This is in agreement with the findings of other authors (see Gould et al. 1986).

**Back titration of (Ca$^{2+}$-Mg$^{2+}$)ATPase**

The reconstitution of (Ca$^{2+}$-Mg$^{2+}$)ATPase in the phospholipid vesicles in the back titration experiments was done as follows: 0.5 mg of EYPC (oxidized or non-oxidized) was dried in a stream of nitrogen and then added to 25 μl of buffer (0.25 mol/l sucrose, 1 mol/l KCl, 5 mmol/l MgATP, 50 mmol/l potassium phosphate, pH 8.0) which contained 0.75 mg of potassium cholate. The content was sealed under dry nitrogen, briefly vortex-mixed and sonicated to clarity (40 s in an 80 kHz sonicating water bath). A (Ca$^{2+}$-Mg$^{2+}$)ATPase aliquot of 32 μg was added and the content was briefly vortex-mixed. The 2 μl aliquots were removed at defined time intervals and the enzyme activity was measured. After 30 min, back titration was performed: 6 μl aliquots of (Ca$^{2+}$-Mg$^{2+}$)ATPase reconstituted into non-oxidized EYPC were transferred into 0.5 mg non-oxidized EYPC or 0.5 mg oxidized EYPC, both solubilized in 25 μl of buffer as described above. In the same way 6 μl aliquots of (Ca$^{2+}$-Mg$^{2+}$)ATPase reconstituted into oxidized EYPC were transferred into 0.5 mg oxidized solubilised EYPC and 0.5 mg non-oxidized solubilised EYPC, respectively. At defined intervals, the 2 μl aliquots were taken and the enzyme activity was measured.

**Estimation of total SH groups**

The amount of SH groups was determined using the slightly modified method of Ellman (1958): 0.5 mg of EYPC in 40 μl buffer was sonicated as described above. An aliquot 32 μg of (Ca$^{2+}$-Mg$^{2+}$)ATPase was added into solubilized EYPC. After a short vortex-mixing the sample was placed into a refrigerator (4°C) for 1 h and subsequently 65 μl of DTNB reagent (50 mmol/l Tris-HCl buffer pH 7.0, 5% potassium cholate, 2 mmol/l DTNB) were added. After mixing and 1 h standing at laboratory temperature the absorbance was measured at 412 nm. The total amount of SH groups was determined using a calibration curve. To construct the calibration curve, glutathione instead of (Ca$^{2+}$-Mg$^{2+}$)ATPase was used in the procedure described above.

**Results**

The kinetics of the EYPC peroxidation, as quantified by the time dependence of the oxidation index $\alpha$, is presented in Fig. 1. The curve is a typical peroxidation curve starting with a lag phase which is necessary for the formation of sufficient amounts of peroxidation reaction centers, followed by a propagation phase characteristic by an increased rate of peroxidation, and ending with a termination phase with the majority of available reaction centers stopping reaction. According to Klein (1970), the observed change in the oxidation index $\alpha$ (from 0.095 to 1.225) corresponds to an increase of total peroxides from 0.5% to 6.25%.

The composition of the EYPC acyl chains before and after a prolonged autoperoxidation, estimated by gas chromatography, is shown in Table 1. It is clear
Figure 1. The time-course of EYPC autooxidation expressed as oxidation index $\alpha$.

Table 1. Composition of the fatty acyl chains (mol %) of EYPC with different oxidation indexes.

<table>
<thead>
<tr>
<th>Chain</th>
<th>$\alpha = 0.095$</th>
<th>$\alpha = 1.225$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>myristic</td>
<td>0.257</td>
</tr>
<tr>
<td>16:0</td>
<td>palmitic</td>
<td>30.162</td>
</tr>
<tr>
<td>16:1n-9</td>
<td>hexadecenoic</td>
<td>0.385</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>palmitoleic</td>
<td>1.527</td>
</tr>
<tr>
<td>17:0</td>
<td>heptadecanoic</td>
<td>0.195</td>
</tr>
<tr>
<td>18:0</td>
<td>stearic</td>
<td>12.386</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>oleic</td>
<td>28.035</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>vaccenic</td>
<td>0.128</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>linoleic</td>
<td>14.058</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>linolenic</td>
<td>0.402</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>eicosenoic</td>
<td>0.237</td>
</tr>
<tr>
<td>20:2n-3</td>
<td>eicosadienoic</td>
<td>0.271</td>
</tr>
<tr>
<td>20:3n-9</td>
<td>eicosatrienoic</td>
<td>0.165</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>dihomo-(\gamma)-linolenic</td>
<td>0.382</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>arachidonic</td>
<td>3.866</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>eicosapentaenoic</td>
<td>0.092</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>docosatetraenoic</td>
<td>0.438</td>
</tr>
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<td>docosapentaenoic</td>
<td>2.029</td>
</tr>
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<td>22:5n-3</td>
<td>docosapentaenoic</td>
<td>0.642</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>docosahexaenoic</td>
<td>4.408</td>
</tr>
</tbody>
</table>
from these data that peroxidation predominantly involves acyl chains with higher numbers of double bonds, i.e. longer chains. The data in Table 1 can be used to calculate an average length of the EYPC acyl chains (number of carbon atoms and the average number of double bonds in these acyl chains) before and after oxidation. This calculation is rather simple: the mean numbers of carbon atoms $n_c$ and double bonds $n_{db}$ are obtained using the equations $n_c = \sum n_{ci} x_i / 100$ and $n_{db} = \sum n_{dbi} x_i / 100$, where $n_{ci}$ is the number of carbon atoms and $n_{dbi}$ the number of double bonds in a particular fatty acyl chain, and $x_i$ is the fraction of this fatty acyl chain (in mol %) given in Table 1. For the oxidation index $\alpha = 0.095$, i.e. for practically non-oxidized EYPC, the mean length of EYPC acyl chains was found to be $n_c = 17.8$ carbon atoms, and the mean number of double bonds $n_{db} = 1.2$; for the oxidation index $\alpha = 1.225$ the mean length of EYPC acyl chains was found to be $n_c = 14.7$ carbon atoms, and the mean number of double bonds $n_{db} = 0.9$. The result for non-oxidized EYPC is in agreement with the data of Requena and Haydon (1975).

**Figure 2.** The dependence of absorbance at 412 nm of a DTNB+glutathione solution on the glutathione concentration in the presence of EYPC liposomes (0.36 mg/ml). Circles, non-oxidized EYPC ($\alpha = 0.095$); squares, oxidized EYPC ($\alpha = 1.225$)

Fig. 2 shows the effect of oxidized EYPC on DTNB estimation of SH groups in glutathione using the same experimental conditions as in experiments with the enzyme described below. It is clearly seen from Fig. 2 that the number of DTNB-accessible groups is not affected by the reconstitution conditions using non-oxidized EYPC ($\alpha = 0.095$), since the value of $A_{412}$ is linearly dependent on glutathione concentration [GSH], and $A_{412} = 0$ at [GSH]=0 as expected. However, the increase in $A_{412}$ in the presence of oxidized EYPC ($\alpha = 1.225$) with the increasing glutathione concentration is shifted towards higher glutathione concentrations.

Fig. 3 shows the kinetics of the absorbance change at 340 nm in the enzyme activity assay system, i.e. the kinetics of NADH $\rightarrow$ NAD$^+$ oxidation in the pre-
Figure 3. The decrease of absorbance at 340 nm in the coupled enzyme assay for the (Ca\textsuperscript{2+}-Mg\textsuperscript{2+})ATPase reconstituted into various oxidized EYPC. Circles, $\alpha = 1.217$; squares, $\alpha = 0.996$; triangles, $\alpha = 0.490$.

Figure 4. The dependence of the (Ca\textsuperscript{2+}-Mg\textsuperscript{2+})ATPase activity reconstituted into various oxidized EYPC, on the oxidation index $\alpha$.

The decrease of absorbance at 340 nm in the assay indicates the presence of reconstituted ATPase, immediately after starting the reactions by adding calcium solution. The decrease in $A_{340}$ is linearly dependent on time till $A_{340} = 0.3$ and then the value of $A_{340}$ levels off. This indicates exhaustion of NADH in the reaction mixture. The enzyme activity was calculated from the slope of the linear part of the $A_{340}$ time dependence. This slope was independent of EYPC concentration in the reaction mixture up to a molar ratio of lipid:enzyme = 1500:1 (not shown), in accordance with other authors (Warren et al. 1974).

Fig. 4 shows the dependence of ATPase activity reconstituted in EYPC vesicles...
on the EYPC oxidation index $\alpha$. It is seen that up to $\alpha = 0.8$ the lipid peroxidation has little effect on the enzyme activity. However, the ATPase activity decreases sharply for $\alpha > 0.8$.

To test the possibility that the change in the enzyme activity is due to the reconstitution procedure itself, check experiments were performed. The (Ca$^{2+}$-Mg$^{2+}$)ATPase reconstituted into non-oxidized EYPC liposomes was re-reconstituted into excess of the same lipid (non-oxidized), and the (Ca$^{2+}$-Mg$^{2+}$)ATPase reconstituted into oxidized EYPC liposomes was transferred into excess of the same
oxidized lipid. It is seen from Fig. 5 that the enzyme activities are not significantly affected by the reconstitution procedure. Therefore, the conclusion could be drawn that the reconstitution procedures are not responsible for the differences in the enzyme activities. It is also noteworthy that the activity does not change in time after the reconstitution or re-reconstitution of the enzyme.

The back-titration (or re-reconstitution) procedure can be used also to test the reversibility of the oxidized EYPC effect on the enzyme activity (see Fig. 6). In these experiments the enzyme reconstituted into non-oxidized EYPC liposomes was transferred into excess of oxidized lipids and the enzyme reconstituted in oxidized EYPC liposomes was transferred into excess of non-oxidized lipids. It is seen that the non-oxidized \(\rightarrow\) oxidized lipid substitution brings about a significant decrease in the enzyme activity from approximately 6.5 U/mg to approximately 1.9 U/mg, which is close to the activity of the enzyme reconstituted directly into oxidized EYPC liposomes. In the second case (oxidized \(\rightarrow\) non-oxidized lipid back-titration) the enzyme activity increased from about 2.4 U/mg to only 3.9 U/mg, which is significantly lower than that of the enzyme reconstituted directly into non-oxidized EYPC liposomes.

If the EYPC bilayer properties were only responsible for the enzyme activity change, the activity of the oxidized \(\rightarrow\) non-oxidized lipid back-titrated enzyme would be expected to be approximately equal to the activity of the enzyme reconstituted directly into non-oxidized lipid. The experimental values are, however, only a half of this value. We conclude therefore from these results that during the (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase reconstitution into oxidized EYPC liposomes protein molecules can be chemically damaged. To test this possibility we determined the amount of SH groups in the reconstituted enzyme. From the results in Fig. 2 obtained with glutathione we calculated the value of molar extinction coefficient for SH groups in DTNB reaction as used in our modification, \(e^1_{412} = 9.700\) 1/mol.cm. Using this value, we determined the amount of SH groups per gram of (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase protein reconstituted in EYPC. Using these values and 110,000 as the value of (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase molecular weight (McLennan et al. 1985) one can calculate the number of SH groups per one molecule of (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase: 9.8 and 3.9 for the enzyme reconstituted into oxidized and oxidized EYPC, respectively. From the results of amino-acid sequencing (McLennan et al. 1985), pure (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase contains 26 cysteine residues. Since some of the cysteine residues can form disulfide bridges, and since the (Ca\(^{2+}\)-Mg\(^{2+}\))ATPase can be partially oxidized before the reconstitution procedure during the isolation and purification, the found value of about 10 free SH groups in enzyme reconstituted into non-oxidized EYPC seems to be reasonable. The relative decrease in the number of SH groups in the enzyme reconstituted into oxidized EYPC to less than half the initial value found for non-oxidized EYPC strongly indicates that this is the protein damage responsible for the irreversible activity decrease.
Figure 7. Activity of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) reconstituted into non-oxidized (circles) and oxidized EYPC (squares) as a function of ATP concentration.

Figure 8. The influence of \(n\)-decane on the activity of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) reconstituted into non-oxidized \((\alpha = 0.095, \text{circles})\) and oxidized EYPC \((\alpha = 1.225, \text{squares})\) as a function of the \(n\)-decane:EYPC molar ratio. \(n\)-Decane was added to the assay mixture from a stock solution (5 mmol/l in methanol) before the 15 min incubation, the maximal added volume was 20 \(\mu l\). The relative activity was obtained by dividing the activity data in U/mg by the activity of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{y-ATPase}\) reconstituted into non-oxidized EYPC with no \(n\)-decane.
Fig. 7 shows the dependence of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) activity on ATP concentration. It can be seen that the enzyme activity increases with the increasing ATP concentration. In the case of the enzyme reconstituted into oxidized EYPC liposomes this increase is slower. It is noteworthy that the enzyme activities are different also in the micromolar range of ATP concentrations.

The influence of \(n\)-decane on the ATPase activity reconstituted into oxidized and non-oxidized EYPC is shown in Fig. 8. The activity of the enzyme in non-oxidized EYPC is initially slightly increased upon the addition of decane. After reaching a flat maximum, the activity decreases. Similar effects were observed also with the enzyme reconstituted into oxidized EYPC. However, the activity enhancement observed was substantially higher than that in non-oxidized EYPC.

**Discussion**

It is clearly seen from Figs. 3-6, that the ATPase activity is affected by the lipids peroxidized before the reconstitution step and that this activity decreases with the increasing oxidation index \(\alpha\). The back-titration experiments (Figs. 5 and 6) clearly demonstrate that the effect of peroxidized lipid on the activity can be separated into a reversible and a non-reversible part. The relative decrease in the numbers of SH groups in the enzyme reconstituted into oxidized EYPC to less than half the initial value found for non-oxidized EYPC strongly indicates that this is the protein damage responsible for the activity decrease. Most of the cysteine residues are located in the cytoplasmic \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) segment containing the nucleotide-binding site, the phosphorylation site and the transduction site of the enzyme (McLennan et al. 1985). Reaction products of lipid oxidation soluble in the aqueous phase (highly hydrated aldehydes such as formaldehyde, acetaldehyde and other shorter chain aldehydes) are known that readily react even at low concentrations with SH groups in enzymes (Esterbauer 1982). Most probably, these products react with the cytoplasmic \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) segment SH groups during the reconstitution of the enzyme in oxidized EYPC. For our experimental conditions, this is supported by results presented in Fig. 2: the number of SH groups in glutathione, which is solubilized in the aqueous phase, is significantly reduced in the presence of oxidized EYPC.

It is well known that the activities of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\text{ATPase}\) vary widely between preparations during storage at low temperature, without any differences in purity obvious on gel electrophoresis. Gould et al. (1986) studied this phenomenon using two different enzyme preparations, one with a high ATPase activity and another with a low activity. In the micromolar range of ATP concentrations, the activities of both preparations were equal and the differences between them were significant at ATP concentrations exceeding \(10^{-5}\) mol/l. They have interpreted this result as a proof of the low-activity preparation not containing large propor-
tions of inactive protein. Moreover, they found that the forward rate constant of the conformation transition $E1'\text{PMgATPH}_2\text{Ca}_2 \rightarrow E2'\text{PMgATPH}_2\text{Ca}_2$ between the enzyme conformation $E1$ (high affinity for Ca and MgATP) and the enzyme conformation $E2$ (low affinity for Ca and MgATP) was higher for high-activity ATPase preparations. We performed similar experiments, results of which are shown in Fig. 7. It can be seen that the enzyme activity increases with the increasing ATP concentration. In the case of the enzyme reconstituted into oxidized EYPC liposomes this increase is slower. It is noteworthy that the enzyme activities are different in the micromolar range of ATP concentrations in contrast to the report by Gould et al. (1986) who observed equal activities for both the low- and the high-activity preparations. This could indicate that the preparation of the $(\text{Ca}^{2+}-\text{Mg}^{2+})$ATPase reconstituted into oxidized EYPC liposomes contains a relatively high proportion of non-active enzyme molecules in comparison with the $(\text{Ca}^{2+}-\text{Mg}^{2+})$ATPase reconstituted into non-oxidized EYPC liposomes. The non-active molecules are probably responsible for the irreversible part of the oxidized EYPC effect on the enzyme activity.

The back-titration experiments (Figs. 5-6) and the experiments with $n$-decane (Fig. 8) strongly indicate that the reversible part of the lipid peroxidation effect is caused by the lipid bilayer properties. Such a biphasic effect of $n$-decane – initial stimulation of the activity followed by a decrease of the activity with the increasing $n$-decane concentration as found in our experiments – has been frequently observed in experiments with the $(\text{Ca}^{2+}-\text{Mg}^{2+})$ATPase reconstituted into lipids with shorter than optimal acyl chains (Johannsson et al. 1981; Lee et al. 1991). The extent of the activity stimulation depends on the lipid acyl chains length: the shorter the chain length the higher the increase in activity. $n$-Decane has been found to bind to the non-annular binding sites in the ATPase molecule or between the ATPase molecules in the ATPase dimer or multimer, relaxing the conformation change of the enzyme brought about by the shorter than optimal acyl chains (Lee et al. 1991). Results in Fig. 8 thus strongly indicate that non-oxidized EYPC bilayer is only slightly thinner than optimal for maximum ATPase activity, while oxidized EYPC bilayer is substantially thinner than needed.

The conclusion that the decrease in thickness of the lipid bilayer could be responsible for the reversible decrease in the ATPase activity reconstituted into oxidized EYPC is further supported by the results of EYPC acyl chain analysis before and after peroxidation (see Table 1). For the oxidation index $\alpha = 0.095$, i.e. for practically non-oxidized EYPC, the mean length of EYPC acyl chains was calculated to be 17.8 carbon atoms, and the mean number of double bonds 1.2. This is only slightly shorter than the optimal acyl chain length 18:1 which is necessary for maximum activity (Johannsson et al. 1981; Lee et al. 1991), and this is why we observed only a slight increase in activity in the presence of decane also for non-oxidized EYPC. However, the oxidation of EYPC brings about a substantial
shortening of acyl chains: for the oxidation index $\alpha = 1.225$ the mean length of acyl chains is 14.7 carbon atoms and the mean number of double bonds is 0.9; probably, this is the reason for a substantial increase in the activity observed in the presence of n-decane. Besides the reduction of the acyl chain length, autooxidation of EYPC brings about a reduction in the number of double bonds in the acyl chains and this could also contribute to the effect of autooxidation on the $(\text{Ca}^{2+}-\text{Mg}^{2+})$ ATPase activity. The role of the number (or position) of the double bonds on the activity of $(\text{Ca}^{2+}-\text{Mg}^{2+})$ ATPase reconstituted into lipid bilayers have not been studied systematically. Based on the reports of Caffrey and Feigenson (1981) and Lee et al. (1986) can be calculated that the increase in activity is about 7 per cent per double bond at the fixed bilayer thickness (4.2–4.3 nm). Since the observed reduction in the number of double bonds in our experiments is maximally 0.3, one would expect about 2 per cent reduction in the $(\text{Ca}^{2+}-\text{Mg}^{2+})$ ATPase activity due to this effect. This is substantially less than observed in our experiments.

In conclusion, we found decreased activity of $(\text{Ca}^{2+}-\text{Mg}^{2+})$ ATPase reconstituted into phosphatidylincholine liposomes when the lipid had been oxidized before the reconstitution procedure. The molecular changes responsible for the decrease of activity are most probably a decrease in the lipid bilayer thickness due to a shortening of lipid acyl chains, and irreversible chemical reactions of the enzyme SH groups with the lipid oxidation products. Since lipid peroxidation is an ubiquitous process in all living system frequently leading to pathological conditions, our results indicate a possible cause for its effect on (trans)membrane enzymes.

References


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