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Partial Characterization of the Inhibitory Effect of Lipid Peroxidation on the Ouabain-Insensitive Na-ATPase of Rat Kidney Cortex Plasma Membranes

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The present work evaluates the effect of lipid peroxidation on the ouabain-insensitive Na-ATPase of basolateral plasma membranes from rat kidney proximal tubular cells as an indirect way to study the lipid dependence of this enzyme. An inverse relationship between lipid peroxidation and Na-ATPase activity was found. This effect was due neither to a change in the optimal K_m of the system for Na^+ nor for the substrate Mg:ATP, nor the optimal pH value of the medium. The optimal temperature value, however, was shifted toward a higher value. There was also an increase of the apparent energy of activation in the region of temperatures above the transition point (20°C) with increase in lipid peroxidation. Peroxidized membranes incubated with phosphatidylcholine from soybean restored their Na-ATPase activity. On the other hand, the Na-ATPase activity was sensitive to oleoly lysophosphatidylcholine. These results suggest that lipid peroxidation might be affecting the Na-ATPase activity through either an increase of peroxidized phospholipids, which might change the membrane fluidity of the lipid microenvironment of the ATPase molecules, or through a direct effect of lysophospholipids released during the lipid peroxidation.

KEY WORDS: Na-ATPase; ouabain-insensitive; lipid peroxidation; basolateral plasma membranes; apparent energy of activation; lysophospholipids; rat kidney cortex.

INTRODUCTION

The lipid peroxidation in biological membrane systems proceeds through a complex process involving rearrangement and destruction of the double bonds of highly unsaturated fatty acids of membrane lipids (Beuge and Aust, 1978; Halliwell and Gutteridge, 1989; Nakamoto *et al.*, 1986; Tappel, 1972; Thomas and Reed, 1990). It is well known that lipid peroxidation of plasma membranes from mammalian cells inhibits the activity of membrane-bound enzymes (Kukreja *et al.*, 1988; Ohta *et al.*, 1989; Scherer and Deamer, 1986). This inhibitory effect can be due to several factors: (1) changes in the optimal conditions to assay the enzyme activity; (2) oxidation of protein residues, i.e., sulfhydryl, methionine, histidine, tryptophan, and tyrosine, which may result in conformational changes of the protein molecules (Torchinsky, 1981); (3) decrease of the membrane fluidity, which affects the conformational changes of several enzymes (Tappel, 1972; Buege and Aust, 1978; Nakamoto *et al.*, 1986; Rice-Evans and Hochstein, 1981; Ohyashiki *et al.*, 1986); (4) inactivation of the enzymes by lysophospholipids, released during lipid peroxidation; (5) interaction of peroxidized fatty acid residues with the protein molecules; (6) formation of Schiff bases and intermolecular cross-links, leading to the formation of oligomer forms of proteins; (7) decrease in the number of active enzymes.

The ouabain-insensitive Na-ATPase (Na-ATPase) is a membrane-bound enzyme that has been

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identified in plasma membrane preparations from several tissues and animals (Moretti *et al.*, 1991; Proverbio *et al.*, 1991). This enzyme has been demonstrated to be a different entity from the Na,K-ATPase (Proverbio *et al.*, 1986, 1989) and to play an important role in volume regulation of kidney cortex cells (Proverbio *et al.*, 1989). Up to now, we have not been able to purify the Na-ATPase in order to study its lipid dependence. Therefore, in the present work we studied the effect of lipid peroxidation on the Na-ATPase activity of basolateral plasma membranes from rat kidney proximal tubular cells, as an indirect approach to evaluate its lipid dependence.

MATERIALS AND METHODS

Chemicals

ATP, ADP, ouabain (strophanthin-G), furosemide, dithiothreitol, lysophosphatidylcholine (oleic acid residue), phenylmethylsulfonylfluoride, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, hexane, and thiourea were purchased from the Sigma Chemical Company; SDS and the protein reagent were obtained from Bio-Rad Laboratories. All other reagents used were of the highest purity obtainable and come from commercial sources.

Preparation of Basolateral Plasma Membranes

Sprague-Dawley rats (3 months old) weighing approximately 250 g were anesthetized with diethyl ether and immediately killed by decapitation. The kidneys were removed, decapsulated, and collected in a medium containing 250 mM sucrose/20 mM Tris-HCl (pH 7.2)/0.5 mM dithiothreitol/0.2 mM phenylmethylsulfonylfluoride, at 4°C. Basolateral plasma membrane fractions were prepared from kidney cortex homogenates following the method described elsewhere (Marín *et al.*, 1986) and stored in a freezer at −20°C until use.

Incubation of Basolateral Plasma Membranes with Phosphatidylcholine

Aliquots of basolateral plasma membranes were treated as follows (Arkhipenko *et al.*, 1983): 1 ml of membrane suspension (100–200 μg proteins) was mixed with 30 μl of 5% (aqueous suspension) phosphatidylcholine from soybeam (66.3% C 18:2, 7.1% C 18:3, 7.1% C 18:1, 3.1% C 18:0, 15.7% C 16:0). The whole suspension was sonicated at room temperature for 30 min and then stored on an ice bath until use.

Lipid Peroxidation of the Basolateral Plasma Membranes

The basolateral plasma membranes were treated as follows: (1) A 250-μl aliquot was poured in a glass vial, placed on ice, and illuminated, from approximately 4 cm distance, by a mineral light (wavelength 254 nm maximum, specified strength 280 μW/cm^2 at 15 cm distance) for different lengths of time (Kako *et al.*, 1988); or (2) the sample was incubated at 37°C for 15 min in a mixture of (mM): 0.1 $FeCl_3$, 1 ADP, 0.1 H_2O_2, and 50 Tris-HCl (pH 7.2) (Halliwell and Gutteridge, 1989).

Lipid Peroxidation Measurements

The amount of lipid peroxidation of the basolateral plasma membranes was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) and conjugated dienes. The TBARS measurements were carried out following the method of Wilburg *et al.* (Wilbur *et al.*, 1949). The values were expressed as nmol of malondialdehyde per mg of proteins (nmol MDA/mg protein). Total lipids were extracted from membrane suspensions following the method of Folch *et al.* (1957). Conjugated dienes were estimated by reading the total lipid extracts in hexane at 233 nm in a spectrophotometer and by using an extinction coefficient of 27,000 $M^{-1}.cm^{-1}$ (Pryor and Castle, 1984).

ATPase Assays

The Na-ATPase activity was assayed following the method already described (Marín *et al.*, 1986). When required, before the assays, the membranes were pretreated with SDS in order to disrupt any membrane vesicle present in the preparation (Forbush, 1982; Marín *et al.*, 1986). The incubation medium contained (final concentrations) 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 100 mM NaCl, 7 mM ouabain, 2 mM $Tris_2$-ATP, and, when required, 2 mM furosemide. The assays were carried out at 37°C for 10 min, stopped, and the liberated inorganic phosphate (P_i) was determined following the method described elsewhere (Forbush, 1982; Marín *et al.*, 1986). The activity was expressed as nmol of P_i liberated per mg protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after stopping the reaction. The protein content of the original suspension was determined by

Table I. Levels of Lipid Peroxidation and ATPase Activities of Basolateral Plasma Membranes Pretreated[d] with UV Light or Fe^{3+}

Condition	TBARS[a]	Dienes[b]	Mg-ATPase[c]	Na-ATPase[c]	Na,K-ATPase[c]
Control	3.75 ± 0.29	2.30 ± 0.09	72 ± 5	178 ± 9	1667 ± 22
UV	10.89 ± 1.26	4.23 ± 0.20	67 ± 6	19 ± 11	1348 ± 18
Fe^{3+}	11.75 ± 1.02	—	65 ± 7	1 ± 2	1362 ± 24

[a]nmol MDA/mg proteins.
[b]nmol hydroperoxides/mg lipids.
[c]nmol P_i/mg protein · min.
[d]The treatment of basolateral plasma membranes with UV light (254 nm) was carried out for 1 h at 4°C. The Fe^{3+} treatment of the membranes was carried out for 15 min at 37°C. TBARS and conjugated dienes were measured following the assays mentioned in Materials and Methods. The values represent the mean ± S.E. (n = 4).

Bradford's method (Bradford, 1976). Na-ATPase activity was calculated as the difference between the P_i liberated in a medium containing Mg^{2+} + Na^+ + ouabain and that liberated in the presence of Mg^{2+} + Na^+ + ouabain + furosemide.

Statistical Analysis

The statistical analysis was done by Student's t-test. All results are expressed as means ± S.E., and n represents the number of experiments performed with different preparations.

RESULTS

Lipid peroxidation of the basolateral plasma membranes was induced by either UV irradiation of the membranes or by the iron-catalyzed Haber–Weiss reaction (Halliwell and Gutteridge, 1989). Table I shows the effect of these treatments on the level of lipid peroxidation of the membranes (as assessed by TBARS and conjugated diene determinations) and on their Mg-, Na-, and Na,K-ATPase activities. Notice that all the assayed ATPase activities are sensitive to lipid peroxidation. However, the Na-ATPase activity shows a higher sensitivity than the Mg- and Na,K-ATPase activities. The Na-ATPase activity is completely inhibited whether the lipid peroxidation is produced by UV irradiation or by the Haber–Weiss reaction. Since the membranes are treated with SDS before the ATPase assays, the shown preferential effect on the Na-ATPase activity might well be due to sensitization of the enzyme to SDS inactivation after the peroxidation procedure. This possibility was tested by studying the ATPase activity of membranes treated with SDS before or after the peroxidation procedure. The results are shown in Table II. It was found that the Na-ATPase activity is 100% inhibited by lipid peroxidation, regardless of the SDS treatment of the membranes before or after UV irradiation.

When the lipid peroxidation is prevented by the presence of thiourea, a hydroxyl radical scavenger (Halliwell and Gutteridge, 1989; Kako *et al.*, 1988; Ohta *et al.*, 1989), or a lipid antioxidant such as Mn^{2+} (Halliwell and Gutteridge, 1989; Kako *et al.*, 1988; May and McCay, 1968; Wills, 1969), the Na-ATPase activity is not affected by the UV irradiation of the plasma membranes (Table III). Interestingly, DTT, a sulfhydryl-reducing agent, is not able to protect the ATPase from UV irradiation (Table III). This might be taken as an indication that SH group oxidation is not involved in the inhibition of the ATPase activity by the lipid peroxidation.

In order to test the possibility that lipid peroxidation might be producing a change in the optimal conditions for the Na-ATPase assay, we evaluated the K_m for Na^+, the K_m for the complex Mg:ATP, and the optimal pH and temperature values for optimal

Table II. Effect of SDS Treatment[a] of Basolateral Plasma Membranes on the Na-ATPase Activity before and after a Peroxidation Procedure

Preincubations	Na-ATPase (nmol P_i/mg protein · min)
None	168 ± 8
SDS	171 ± 8
UV	8 ± 6
UV + SDS	14 ± 9
SDS + UV	17 ± 12

[a]The treatment of basolateral plasma membranes with SDS was carried out as described in Materials and Methods. UV light (254 nm) treatment was carried out for 1 h at 4°C. The values represent the mean ± S.E. (n = 4).

Table III. Na-ATPase Activity and Levels of Lipid Peroxidation of Basolateral Plasma Membranes Treated[f] under Different Experimental Conditions

Condition	TBARS[a]	Dienes[b]	Na-ATPase[c]
Control	3.57 ± 0.36	2.37 ± 0.08	167 ± 11
UV + Mn^{2+}	3.33 ± 0.84[d]	—	177 ± 8[d]
UV + thiourea	5.63 ± 0.62[e]	1.95 ± 0.26[d]	172 ± 12[d]
UV + DTT	12.34 ± 0.88[e]	4.35 ± 0.16[e]	21 ± 4[e]

[a] nmol MDA/mg proteins.
[b] nmol hydroperoxides/mg lipids.
[c] nmol P_i/mg protein · min.
[d] P not significant, when compared with control values.
[e] $P < 0.001$, when compared with control values.
[f] The treatment of basolateral plasma membranes with UV light (254 nm) was carried out for 1 h at 4°C in the presence or absence of 1 mM DTT, 0.4 mM Mn^{2+}, or 4 mM thiourea. TBARS and conjugated dienes were measured following the assays mentioned in Materials and Methods. The values represent the mean ± S.E. ($n = 4$).

Table V. Apparent Energy of Activation[c] of the Na-ATPase for Two Ranges of Temperature (10–20°C and 20–47°C) for Basolateral Plasma Membranes Treated or Not Treated with UV Light or UV Light + Thiourea.

	Energy of activation (kcal/mol)	
Condition	10–20°C	20–47°C
Control	17.52 ± 0.28	9.36 ± 0.19
30 min UV	17.48 ± 0.31[a]	11.80 ± 0.15[b]
60 min UV + thiourea	17.35 ± 0.23[a]	9.32 ± 0.21[a]

[a] P not significant, when compared with control values.
[b] $P < 0.001$, when compared with control values.
[c] The energy of activation (E_a) was calculated from the expression

$$E_a = -m \cdot 2{,}303R$$

where m is the slope of the Arrhenius plot, and R is the gas constant (1,987 cal · mol^{-1} · $°K^{-1}$). The values are expressed as means ± S.E. ($n = 4$).

ATPase activity, for membranes irradiated or not irradiated with UV during 30 min, conditions under which the Na-ATPase activity is inhibited by approximately 50%. The results of these experiments are shown in Table IV. For the tested parameters, only the optimal temperature value changed from 47 to 49°C with the UV treatment. The higher optimal temperature might indicate a decreased membrane fluidity. If this were the case, a change might be expected in the activation energy (E_a) of the ATPase. This possibility was tested by determining the Na-ATPase activity of membranes, treated or not treated with UV light, as a function of the temperature of the incubation medium. The UV light treatment was carried out for 30 min in order to obtain membranes with the Na-ATPase activity inhibited by about 50%. As a second control, we tested the ATPase activity of membranes treated for 60 min with UV light in the presence of thiourea, a well-known hydroxyl radical scavenger (Halliwell and Gutteridge, 1989; Kako *et al.*, 1988; Ohta *et al.*, 1989), in order to protect the ATPase activity. In all the cases, we estimated the apparent energy of activation by means of the Arrhenius plot, which for the ATPase activities shows two slopes with a transition point at around 20°C (Charnock *et al.*, 1971, 1975). Table V shows the results of this experiment. It can be noticed that in the region above 20°C, the membranes treated with UV during 30 min show a value of activation energy higher than the control membranes. This result can be taken as an indication of an effect of the lipid peroxidation on the basolateral plasma membrane fluidity, as already demonstrated for several membranes (Tappel, 1972; Buege and Aust, 1978; Nakamoto *et al.*, 1986; Rice-Evans and Hochstein, 1981; Ohyashiki *et al.*, 1986).

On the other hand, if the lipid microenvironment modulates the activity of the Na-ATPase, the peroxidized phospholipids might be responsible for its inhibition. Hence, if one can exchange the peroxidized phospholipids by nonperoxidized ones, the Na-ATPase activity could be restored. This possibility was tested by incubating basolateral plasma membranes, treated or not treated with UV, with phosphatidylcholine from soybean, enriched in unsaturated fatty acid residues (66.3% C 18:2, 7.1% C 18:3, 7.1% C 18:1), and then assayed for Na-ATPase activity and lipid peroxidation (TBARS). The results of this experiment are shown in Table VI. It can be seen that incubation of the peroxidized membranes with

Table IV. Partial Characterization of the Na-ATPase of Control or Experimental Rat Kidney Basolateral Plasma Membranes[a]

Parameter	Control membranes	UV-treated membranes
K_m^{Na} (mM)	8.0	8.0
$K_m^{Mg:ATP}$ (mM : mM)	1.8 : 0.3	1.8 : 0.3
Optimal temperature	47°C	49°C
Optimal pH	6.9	6.9

[a] The treatment of basolateral plasma membranes with UV light (254 nm) was carried out for 30 min at 4°C. The optimal temperature value was determined at a constant pH of 7.2.

Table VI. Effect of the Incubation of Basolateral Plasma Membranes, Treated[f] or Not Treated with UV, with Exogenous Phosphatidylcholine (PC) on Na-ATPase Activity and TBARS

Pretreatments			
(A)	(B)	Na-ATPase[a]	TBARS[b]
1. None	None	182 ± 14	3.44 ± 0.22
2. None	PC	195 ± 12[c]	2.65 ± 0.12[d]
3. UV	None	7 ± 5[e]	11.32 ± 1.24[e]
4. UV	PC	123 ± 14[e]	7.51 ± 0.83[e]

[a] nmol P_i/mg protein · min.
[b] nmol MDA/mg protein.
[c] P not significant, when compared with values of row 1.
[d] $P < 0.01$, when compared with values of row 1.
[e] $P < 0.001$, when compared with values of row 1.
[f] The treatment of basolateral plasma membranes with UV light (254 nm) was carried out for 30 min at 4°C. Incubation of basolateral plasma membranes with exogenous phosphatidylcholine from soybean and TBARS assay were carried out as described in Materials and Methods. The values represent the mean ± S.E. (n = 6).

phosphatidylcholine partially restored the Na-ATPase activity. A concomitant diminution of the level of peroxidation of the membranes was also found.

Another possibility would be the presence of lysophospholipids released as lipid peroxidation proceeds. The lysophospholipids are known as inhibitors of the Na,K-ATPase activity (Corr *et al.*, 1984; Pitts and Okhuysen, 1983), and hence they could also inhibit the Na-ATPase activity. In order to test this possibility, we evaluated the effect of different amounts of oleoyl lysophosphatidylcholine (Lyso PC) on the Na-ATPase activity. The results are shown in Fig. 1. Notice that the Na-ATPase activity is sensitive to Lyso PC, being 100% inhibited by approximately 1100 nmol of Lyso PC/mg proteins.

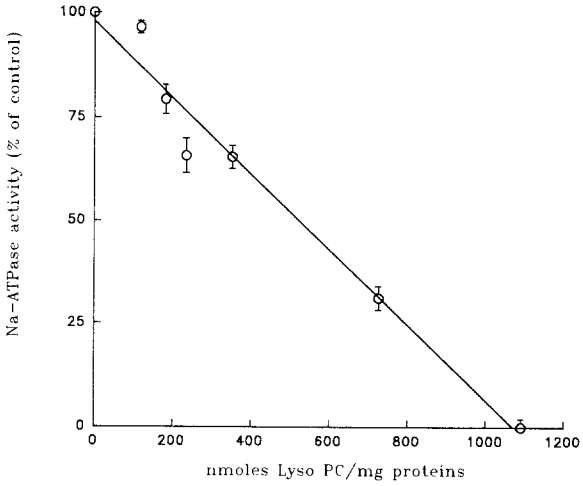

Fig. 1. Effect of oleoyl lysophosphatidylcholine (Lyso PC) on Na-ATPase in basolateral plasma membranes. The membranes were preincubated for 30 min at room temperature with various concentrations of Lyso PC, as indicated. The Na-ATPase activity was then determined as described in Materials and Methods. The activity value obtained in the absence of Lyso PC (183 ± 9 nmoles P_i/mg protein · min) was taken as 100%. The values represent the mean ± S.E. (n = 9)

DISCUSSION

The present results show that the Na-ATPase activity possesses a higher sensitivity to lipid peroxidation than that of the Mg- and Na,K-ATPases (Table I). This higher sensitivity cannot be explained as due to SDS inactivation after the peroxidation procedure (Table II). The fact that a hydroxyl radical scavenger, like thiourea, and an antioxidant, like Mn^{2+}, prevent the inhibition of the Na-ATPase by the UV treatment (Table III) indicates a clear relationship between ATPase activity and lipid peroxidation. Considering that lipid peroxidation is a very complex process, it is likely that the inhibition of the Na-ATPase activity is the result of a combination of direct and indirect effects on the lipid microenvironment as well as on the protein molecule itself. In this regard, it is important to discuss the possible mechanisms of the modifying action of the lipid peroxidation on the Na-ATPase.

Changes in the Optimal Conditions for the Na-ATPase Assay

If lipid peroxidation is affecting, directly or indirectly, the protein, the optimal conditions to assay the Na-ATPase might be displaced. This does not seem to be the case, since we found no changes in the K_m for Na^+, nor for Mg : ATP, nor for the optimal pH value of the incubation medium for the ATPase assay (Table IV).

Oxidation of SH Groups

It is well known that the SH groups of the membrane proteins are readily oxidized under the peroxidation conditions utilized in the present work (Kako *et al.*, 1988). On the other hand, as shown in Table III, DTT, a sulfhydryl-reducing agent, was not able to protect the Na-ATPase activity from UV irradiation. It seems then that the Na-ATPase does not depend on

the SH groups for its activity. This conclusion is supported by the fact that the NEM treatment of the basolateral plasma membranes does not affect the activity of this enzyme (data not shown). Similarly, the Ca-ATPase activity of rat liver and intestinal membranes has been shown to be independent of the status of the SH groups (Lin, 1985; Ohta *et al.*, 1989).

Diminution of Membrane Fluidity

The change of the apparent energy of activation in the region above 20°C (Table V) suggests that the inhibition of the ATPase activity may occur through changes in membrane fluidity. This explanation is based on the known fact that lipid peroxidation of plasma membranes produces a decrease of their fluidity (Tappel, 1972; Beuge and Aust, 1978; Nakamoto *et al.*, 1986; Rice-Evans and Hochstein, 1981; Ohyashiki *et al.*, 1986). However, this change is too small to be considered the main mechanism responsible for the inhibition of the ATPase.

Inhibitory Effect of Lysophospholipids Released During Lipid Peroxidation

As lipid peroxidation proceeds, several lysophospholipids are released, which have a detergent-like activity, contributing to increase membrane disruption and further lipid peroxidation (Halliwell and Gutteridge, 1989). In this regard, lysophospholipids are reported to inhibit the activity of several enzymes including the Na,K-ATPase (Oishi *et al.*, 1990). The results shown in Fig. 1 clearly indicate that exogenous lysophospholipids are able to inhibit the Na-ATPase. Consequently, lysophospholipids released during lipid peroxidation of the basolateral plasma membranes could be, at least partially, responsible for the inhibition of the Na-ATPase activity. In this regard, we have found a significant diminution of the phospholipid content of the basolateral plasma membranes after UV irradiation (data not shown).

Interaction of Peroxidized Fatty Acid Residues with Na-ATPase Molecules

The lipid microenvironment where the Na,K-ATPase protomers are inserted is widely known to participate in the modulation of the activity of this enzyme (Cornelius and Skou, 1984; Marcus *et al.*, 1986; Roelofsen, 1981). If this is the case for the Na-ATPase, it is clear that peroxidation of the fatty acid residues of the lipid microenvironment might be participating in the inhibition of this enzyme. The results shown in Table VI are in agreement with this idea. The fact that we were able to restore partially the Na-ATPase activity of UV-treated membranes with phosphatidylcholine lead us to propose that this treatment might be exchanging peroxidized phsopholipids within the membranes by nonperoxidized ones or it might be restoring the membrane structure. Similar results with exogenous phospholipids have been reported with peroxidized membranes and the Ca-ATPase activity (Arkhipenko *et al.*, 1983).

Formation of Oligomer Forms of Na-ATPase Molecules

Some of the by-products of lipid peroxidation, e.g., malondialdehyde, are cross-linking agents of membrane components (Dillard and Tappel, 1984). The lipid peroxidation, through the formation of Schiff bases, would induce intermolecular cross-links, with the formation of oligomer forms of proteins. We cannot disregard this possibility as well as any damage to aminoacyl residues of the protein.

Decrease in the Number of Active Enzymes

Unfortunately we do not have a specific binding assay to evaluate the number of ATPase molecules. Therefore, we cannot disregard this possibility.

The present results might have important implications. Preliminary experiments have shown that ischemia-reperfusion of the rat kidney produces a complete inhibition of the Na-ATPase activity of the basolateral plasma membranes, with a concomitant increase in the level of lipid peroxidation of those membranes (data not shown). Since this enzyme has been reported to be involved in the active regulation of the volume of these cells (Proverbio *et al.*, 1989), its inhibition as a result of ischemia-reperfusion could be contributing to cell swelling. It is important to mention that the results shown in Table I indicate the higher sensitivity of this enzyme toward the lipid peroxidation when compared with the Mg- and Na,K-ATPase activity.

From the results presented it may be proposed that the Na-ATPase of basolateral plasma membranes possesses a strong dependence of its lipid microenvironment, which seems to modulate the activity of this enzyme.

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