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ACTIVATION OF (Na^+ + K^+)-DEPENDENT ATPase BY LIPID VESICLES OF NEGATIVE PHOSPHOLIPIDS

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Summary

1. Kidney (Na^+ + K^+)-stimulated ATPase was depleted of phospholipids by extraction with lubrol and inserted in lipid structures of known composition. Both ouabain-sensitive ATPase and phosphatase reactions could be partially restored by lipid replacement.

2. Lipid vesicles of natural and synthetic negative phospholipids proved to be effective. The low activity of uncharged liposomes was increased when negative charges were included into the bilayer structure.

3. Reactivation by negative phospholipids was accompanied by spontaneous re-assembly of a stable lipid · protein complex. By contrast, the interaction of lipid deficient ATPase complex with uncharged lamellae was possible only after sonication of lipid-protein suspension. Reactivation did not ensue.

4. The ouabain-sensitive ATPase reactivated by synthetic dioleoylphosphatidylglycerol yielded curvilinear Arrhenius plots. The same pattern was seen with the original undepleted microsomal preparation. A discontinuity close to the temperature of fluid-order transition was found with dimyristoyl phosphatidylglycerol.

5. It is concluded that reassembly of lipid-deficient (Na^+ + K^+)-stimulated ATPase requires the addition of diacylphospholipids with fluid acyl-chains and negatively charged polar heads able to assemble in an expanded lamellar configuration.

Introduction

Deoxycholate [1] and lubrol [2,3] are currently used to deplete (Na^+ + K^+)-stimulated ATPase of phospholipid content. Treatment with these detergents offers the advantage of almost complete depletion without total inactivation of enzyme since substitution of natural lipids with amphiphilic molecules

probably preserves the correct subunit association. In this respect lubrol proved to be safer and more effective than deoxycholate [3]. The use of lubrol has been extensively studied[2—6]. Nevertheless, the evidence is still incomplete in regard to the possibility of correlation between lipid-induced reactivation and the physical properties of the reactivating lipid dispersion. Such a study is a necessary condition for subsequent investigations on the role of lipid configuration, distribution of different phospholipids within bimolecular arrangments and lateral molecular motions, as determined by the structure of phospholipid acyl-chains and polar head. These properties of the phospholipid lamellar phase constitute a coherent and detailed picture relevant to membrane structure and function [7]. The purpose of this paper is to study the reactivation of (Na^+ + K^+)-stimulated ATPase with respect to the composition of added phospholipid vesicles, the incorporation of protein in the lipid structure and the thermal transition of the lipid environment.

Materials and Methods

A commercial preparation of Lubrol W (Sigma) was used without further purification. At a 10% concentration it yielded a cloudy solution. A clear solution could be obtained by dissolving the detergent in acetone, precipitating and washing with cold pretroleum ether. The effectiveness of lubrol was, however, slightly decreased by this procedure. Dioleoyl phosphatidylglycerol and dimyristoyl phosphatidylglycerol were prepared from the corresponding phosphatidylcholine [8]. Other purified phospholipids were purchased from General Biochemicals. The purity of phospholipids was tested by two dimensional thin layer chromatography as described previously [2]. *p*-Nitrophenylphosphatase activity was tested at 37° C under the conditions described by Robinson [9]. Bound phospholipids were estimated by the method of Bartlett [10]. ATPase activity determination and other methods were as reported previously [2].

Preparations of phospholipid-dependent (Na^+ + K^+)-ATPase

The original procedure [2] was slightly modified to be adaptable for kidney preparations. This simplified version, however, proved to be equally effective for bovine-heart preparation. Microsomal fractions were obtained from kidney-cortex of guinea-pig, and pig as described by Kimelberg and Papahadjopoulos [11]; NaI treatment was as reported by Uesugi et al. [12]. Preparation of microsomal fraction from bovine-heart and NaI treatment were as reported previously [2], omitting the final treatment with sonic oscillations. These materials were stored at —40° C until needed.

The first extraction with lubrol was as follows: the NaI treated preparation was thawed and suspended in 35% (w/v) glycerol, 5 mM β-mercaptoethanol, 2.5 mM ATP, 25 mM sucrose, 0.1 mM EDTA. A 10% (w/v) solution of lubrol was then added with continuous stirring at 0° C to yield a concentration of 10 mg detergent/mg protein; the final concentration of protein was 2 mg/ml. The mixture was sonicated at 0° C for a total time of 90 s (3 bursts of 30 s each with a Biosonik III apparatus at maximum output). Sonication was essential to get effective solubilization by lubrol and in the original procedure was

performed before detergent treatment. Simultaneous treatment with sonic oscillations and lubrol was also used by Wheeler et al. [3].

After 30 min centrifugation in the cold at 105 000 × *g* (40 000 rev./min, Spinco, No. 40 rotor), solid ammonium sulfate was added to the supernatant fluid to yield 35% saturation. After 20 min centrifugation at the same speed the quasi-solid floating layer was collected, dispersed in 0.2 M NaCl, sedimented 90 min at 150 000 × *g* (50 000 rev./min, No. 50 rotor) and washed with 0.6 M NaCl to avoid interference by lubrol in the Lowry value of protein determination. The second treatment with lubrol was an equilibration step: 10 mg detergent/mg protein were added to the enzyme preparation (2 mg/ml) suspended at 0° C in the ATP, glycerol, sucrose, EDTA, β-mercaptoethanol solution. After 15 min stirring, solid ammonium sulfate was added and the suspension centrifuged. The floating layer was collected, washed with 0.2, 0.6, 1 M NaCl and the final sediment resuspended in 0.25 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4) and stored at −40° C in small aliquots. The yield, in terms of protein, was 16—19% of NaI-treated microsomal fractions.

Reassembly of delipidated enzyme

Two procedures were used: (a) the dry phospholipids were suspended in 0.25 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4) and sonicated 3 min above the transition temperature at a concentration of approx. 5 mg/ml. Liposomes of phosphatidylcholine required a longer sonication to yield vesicles of small diameter. 200 μg enzyme complex were added to 0.35 ml of 140 mM sucrose, 6 mM Tris · HCl, 0.6 mM EDTA (pH 7.4) containing the sonicated liposomes. After 10 min at 37° C the medium was completed with a stock solution containing the components needed for determination of ATPase or phosphatase activities. (b) To estimate the amount of phospholipids bound as a stable complex with the protein, 2—3 mg enzyme complex were added to 0.8 ml of 10 mM Tris · HCl, 1 mM EDTA containing the liposomes sonicated as above. After 10 min at 37° C the suspension was transferred to the surface of 12 ml of 0.31 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4) and centrifuged in the cold for 30 min at 97 000 × *g* (30 000 rev./min, SW 36 rotor). The reconstituted enzyme with bound phospholipids sedimented to the bottom of the centrifuge tube, whereas the unbound phospholipid remained afloat above the concentrated sucrose solution. The sediment was washed once with 0.2 M NaCl, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4) and once with 0.25 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4).

Results

In ATPase isolated from pig or guinea-pig kidney about 0.07 μmol phospholipid remained per mg protein (range, 0.05—0.1 μmol phospholipid/mg protein in 6 preparations), representing 90—95% depletion of phospholipids. The values for bovine-heart preparation were in agreement with the previous report [2] (approx. 93% phospholipid depletion). Parallel with the lipid depletion, the ouabain-sensitive ATPase activity was reduced by 95—100% and the ouabain-sensitive phosphatase activity by 85—95%. Addition of lipids produced simultaneous restoration of both activities. The degree of reactivation was

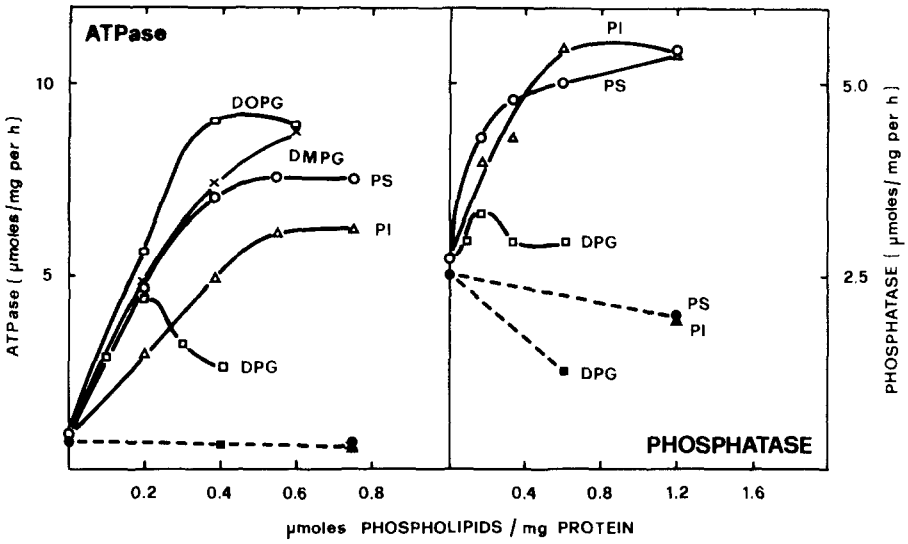

Fig. 1. Reactivation by anionic liposomes of natural and synthetic phospholipids. The guinea-pig kidney preparation was reconstituted with sonicated phospholipids according to the first procedure of Materials and Methods, Open symbols and continuous line, without ouabain. Closed symbols and dotted line, with 0.1 mM ouabain. DOPG, synthetic dioleoyl phosphatidylglycerol; DMPG, synthetic dimyristoyl phosphatidylglycerol; PS, bovine-brain phosphatidylserine; PI, bovine-brain phosphatidylinositol; DPG, bovine-heart diphosphatidylglycerol. The ATPase activity of original, NaI-treated microsomal fraction before phospholipid depletion was 23.4 µmol/mg per h (inhibition by ouabain, 90%); the phosphatase activity was 11.9 µmol/mg per h (inhibition by ouabain, 64%).

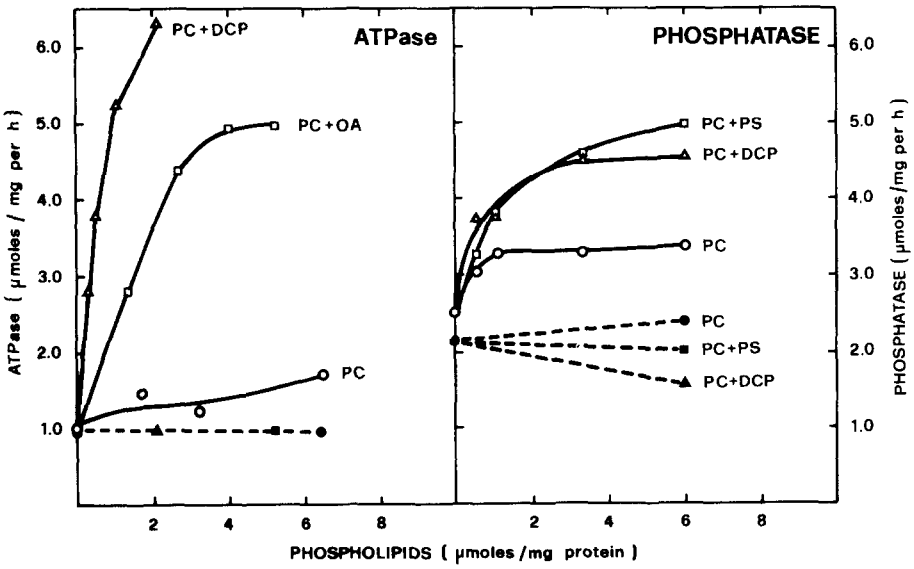

Fig. 2. Role of negative charges. The guinea-pig kidney preparation was reconstituted with sonicated phospholipids according to the first procedure of Materials and Methods. Mixed liposomes were prepared with bovine-brain phosphatidylcholine (PC) and dicetylphosphate (DCP) (78 : 22 mol : mol) or oleic acid (OA) (65 : 35 mol : mol) by dissolving lipids in chloroform. The solvent was evaporated under nitrogen and the dry material sonicated in 0.25 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4). Open symbols and continuous line, without ouabain. Closed symbols and dotted line, with 0.1 mM ouabain. Control experiments with the amounts of oleic acid or dicetylphosphate added with phosphatidylcholine (0.1—1.8 µmol/mg protein) showed that no activation was produced by these compounds.

70—90% in preparations from porcine kidney and bovine heart and only 30—40% from guinea-pig kidney. In spite of its lover yield of reactivation, the guinea-pig kidney preparation was selected for further studies, since in this case the NaI treatment of the microsomal fraction was very efficient in removing the ouabain-insensitive activity. The phospholipid induced reactivation was abolished by ouabain, indicating that in all instances the ouabain-sensitive activity originated from a phospholipid-dependent stimulation in agreement with the current view of a phospholipid dependent sodium pump [13,14].

Fig. 1 shows the stimulation of both ATPase and phosphatase activities of the lipid deficient enzyme by the addition of sonicated anionic liposomes of single purified phospholipids. Significant reactivation was found with natural acidic phospholipids phosphatidylserine, phosphatidylinositol and diphosphatidylglycerol as well as with synthetic dioleoyl phosphatidylglycerol and dimyristoyl phosphatidylglycerol. The ratio between lipid and protein was critical. This was particularly apparent in the case of diphosphatidylglycerol that produced stimulation only at low concentrations (see also ref. 2). The liposomes induced reactivation of phosphatase activity also augmented the sensitivity to ouabain. The increase of ouabain-effect might be related to the difficulty of getting complete ouabain effect on the lipid-poor system in the absence of sodium (cf. ref. 5).

In contrast with effective anionic liposomes, uncharged vesicles of bovine-brain phosphatidylcholine showed little or no activity on the ATPase and phosphatase reactions (Fig. 2). Similar observations were made with the rabbit kidney enzyme [3]. The uncharged lamellae of phosphatidylcholine gained activating effect after the introduction of negative charges by incorporation of dicetylphosphate or oleate, which by themselves did not produce stimulation. Phosphatidylserine could substitute for dicetylphosphate or oleate. It can be appreciated that liposomes in which negative lipids were diluted by uncharged phosphatidylcholine, were effective only in considerably higher amount than were liposomes that consisted of a single anionic phospholipid. Introduction of positively charged ethylhexadecyldimethylammonium into phosphatidylcholine liposomes did not improve the reactivation.

The lack of activation by uncharged lamellae could be ascribed either to an inability to interact with the protein or to an inefficient activation of the reconstituted lipid · protein complex. Previous experiments [2] have indicated that when the phospholipid-deficient preparation was reconstituted with a mixture of neutral and acidic phospholipids a preferential binding of charged species was manifest. In order to determine the extent of incorporation of protein in the lipid structure the procedure indicated in Table I was adopted. The passage through a sucrose solution of appropriate concentration offered an efficient and rapid system for separating unbound vesicles as well as for retaining significant activity in the reconstituted system. An average value of 60% protein was recovered in the final sediment together with 60% initial ouabain-sensitive activity. Loss of activity can in part be prevented performing the centrifugations at room temperature. Active vesicles of negative phospholipids reconstituted a stable lipid · protein complex with a final amount of phospholipids similar to that of the original membrane. By contrast, inactive

TABLE I

INCORPORATION OF PHOSPHOLIPIDS IN REACTIVATED ATPase

2 mg lipid-depleted ($Na^+ + K^+$)-ATPase (0.1 μmol residual phospholipids/mg protein) were incubated with phospholipid vesicles as described under Materials and Methods and layered on top of 0.31 M sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 7.4). After 30 min centrifugation at 97 000 × g the sediments were collected and washed twice. The increase of phospholipid content and the ouabain-sensitive ATPase activity were determined in the final sediments. Where indicated, incorporation of ATPase into phospholipid vesicles was enhanced by 2 min sonication at room tempterature (Biosonik III, minimum setting of instrument with a small probe). The sonicated mixture was centrifuged as above. Mean values of separate experiments. Ranges are also indicated. All phospholipids were from bovine-brain.

Phospholipid added (μmol/mg)	Number of determinations	Phospholipid bound (μmol/mg)	Ouabain-sensitive ATPase activity (μmol/mg per h)
1.2 μmol phosphatidylserine	5	0.64 (0.5—0.9)	3.0 (2.4—3.7)
1.2 μmol phosphatidylinositol	2	0.75 (0.6—0.9)	1.9 (1.6—2.2)
4.0 μmol phosphatidylcholine	5	0.12 (0.1—0.2)	0.3 (0.0—0.6)
4.0 μmol phosphatidylcholine + sonication	3	0.90 (0.8—1.0)	0.4 (0.0—0.9)
4.0 μmol phosphatidylcholine with 20% dicetyl phosphate	3	0.90 (0.8—1.0)	3.5 (1.4—5.3)
4.0 μmol phosphatidylcholine with 20% dicetyl phosphate + sonication	2	1.5 (1.5—1.5)	3.2 (2.5—3.9)

uncharged lamellae failed to interact efficiently with protein. In this case incorporation of protein could be forced by sonication of lipid/protein mixture but reactivation did not follow. Incorporation spontaneously occurred upon the introduction of 20% dicetylphosphate into the bilayer of phosphatidylcholine proving the need for negative charges for both reconstitution and activation. Thin-layer chromatography analysis confirmed that phosphatidylcholine was bound together with dicetylphosphate under these conditions. Appropriate controls (last line) excluded that sonication during the reconstitution procedure inactivated the enzyme preparation. The amount of bound phospholipid was also increased in this case. The findings indicating that reactivation and regeneration of the lipid · protein complex coincided, and that synthetic phosphatidylglycerols of known fluid-order transition temperatures reactivated the enzyme, made it possible to study the effects of phospholipids in relation to their thermal transition. Such study has only been done on deoxycholate treated enzyme [15]. Fig. 3 presents Arrhenius plots of the original and reactivated preparations from guinea-pig kidney and bovine heart. Results are also included here from a NaI treated microsomal fraction of the bovine brain. The ouabain-sensitive activity of NaI-treated microsomal fractions was better described by curved lines perhaps reflecting multiple breaks [22]. Curved Arrhenius kinetics are not unusual with membrane bound enzymes and were reported also with ($Na^+ + K^+$)-stimulated ATPase [23]. Their possible significance is discussed in a recent study [24]. Reconstitution of lubrol-treated enzymes with dimyristoyl phosphatidylglycerol gave rise to a dis-

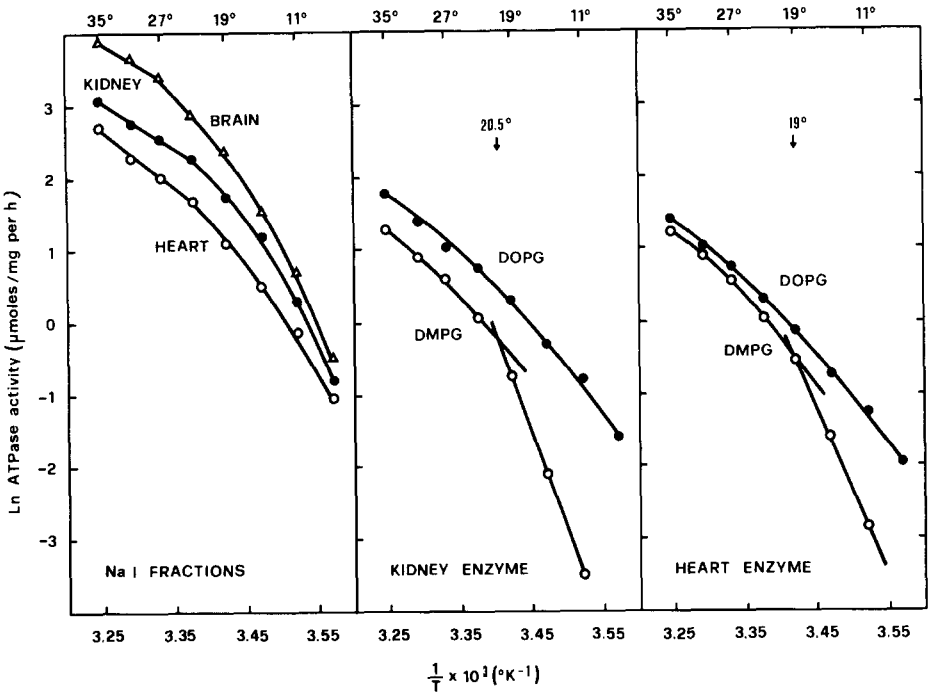

Fig. 3. Arrhenius plots with synthetic phosphatidylglycerols. Brain microsomal fraction treated with NaI [11,12] was prepared from pooled guinea-pig brains. The delipidated fractions were reconstituted at 37°C with phospholipids (0.7 μmol/mg protein) according to the first procedure described in Materials and Methods. A 0.2 ml aliquot was added to 0.7 ml of incubation medium which did not contain ATP and Mg^{2+} and which was equilibrated at different temperatures. After about 5 min further thermoequilibration, the reaction was started with 0.1 ml of a 30 mM ATP and Mg^{2+} solution. To distinguish between $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase determinations were performed also in the presence of a large excess of ouabain (0.2 mM) the effect of which was not influenced by temperature changes [15—20]. The small ouabain-insensitive activity was subtracted. Possible influence of pH variations [21] was avoided adjusting the pH of Tris · HCl buffer at 7.4 at each temperature. Incubation, NaI-treated fractions 20 min; reconstituted enzyme, 40 min. DOPG, dioleoyl phosphatidylglycerol. DMPG, dimyristoyl phosphatidylglycerol.

continuity at about 20°C whereas with the unsaturated dioleoyl phosphatidylglycerol a sharp discontinuity in the Arrhenius plot was not recorded. In the presence of either one of the two phospholipids, the temperature effect was curvilinear above the thermotropic phase transition, as it was in the original undepleted preparations. With deoxycholate-treated enzyme [15] a discontinuity was observed at 20.2°C with dimyristoyl phosphatidylglycerol whereas dioleoyl phosphatidylglycerol gave a linear Arrhenius plot. However, the curvilinear tendency was evident also in this case.

Discussion

The results presented demonstrate the reactivation of lipid-deficient $(Na^+ + K^+)$-stimulated ATPase upon the addition of various diacylphospholipids that assemble in a negatively charged lamellar configuration.

The findings of this paper do not exclude that in the intact membrane phosphatidylserine may be the specific activator of transport ATPase [13,25] and

that its carboxyl group may be implicated when high rate of cation transport is taking place. However no specific requirement for phosphatidylserine has been found in the limited reconstitution of enzymatic activity in lipid depleted preparations. Differences were recorded among the active, single phospholipids used in the reconstitution but these correlated with the general properties of bilayer structure, rather than the existence of specific chemical groups in the individual molecules. A lamellar organization with appropriate density of negative charges is sufficient to allow a correct interaction between lipids and protein and activate the ATPase. The requirement for negative charges was particularly impressive when the reassembly of lipid · protein complex was examined. Apparently, negative charges are needed for both interaction of lamellae with protein and reactivation of enzyme. The incorporation of protein into a preformed bilayer structure may be facilitated by negative charges through electrostatic interaction with protein or by producing lateral expansion of phospholipid molecules within the lamellae. Further studies are required to explore these possibilities. The reaction of transport ATPase follows the interaction between protein and a bilayer structure bearing negative charges. Therefore, the conflicting results (reviewed in refs. 13, 14), in regard to the effect of various phospholipids on lipid deficient ATPase preparations, may find appropriate explanation because all long chain diacylphospholipids assemble in a bimolecular arrangement. The effectiveness of high amount of neutral phospholipids reported in some studies might originate from negative charges introduced with fatty acids liberated during preparations of lipid vesicles, whereas observations on the lack of stimulation by some negative phospholipids may be the consequence of inactivation in labile preparations requiring high lipid : protein ratio to be activated.

The observation that reactivation of ouabain-sensitive ATPase with dimyristoyl phosphatidylglycerol was dependent on the phase properties of this phospholipid is in line with the current view that membrane-bound enzymes requires a fluid state of membrane lipids to be fully active (cf. ref. 7). This result also indicates that the emergence of ouabain-sensitive activity was not due to residual low amount of phospholipids nor to removal of inhibitory concentration of lubrol left in the preparation. The Arrhenius kinetics obtained with lubrol-enzyme are in agreement with deoxycholate enzyme [15] and confirm that phase transition of bilayer structure affects the activation energies for $(Na^+ + K^+)$-dependent ATPase that, as an enzyme involved in the transport of cations across membranes, is expected to possess extensive conformational mobility.

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