Biochimica et Biophysica Acta, 556 (1979) 479—489

BBA 78504

PRESSURE EFFECTS ON LIPID-PROTEIN INTERACTIONS IN ($Na^+ + K^+$)-ATPase

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(Received January 12th, 1979)

Key words: Pressure effect; Lipid-protein interaction; ($Na^+ + K^+$)-ATPase

Summary

The ($Na^+ + K^+$)-stimulated ATPase activity decreases with increasing pressure and a plot of the logarithm of the activity versus pressure shows a change in slope at a defined breakpoint pressure (P_b). The value of P_b increases linearly with increasing temperature. A dT/dP value of 27.7 ± 0.4 (S.D.) K/1000 atm is obtained. This is in very good agreement with the pressure shift for the melting transitions in phospholipids and aliphatic chains. This strongly indicates that an aliphatic chain melting process is involved in the breakpoint in the Arrhenius plot and pressure dependence of ($Na^+ + K^+$)-ATPase. The *p*-nitrophenyl phosphatase activity of this enzyme also decreases with pressure. In this case the plot of the logarithm of the activity versus pressure is linear without a break-point. The temperature dependence for ($Na^+ + K^+$)-ATPase was also studied in the presence of fluidizing drugs: desipramine and benzylalcohol. The presence of these drugs had no effect on the inflection point in the Arrhenius plot.

Introduction

The existence of a non-linear Arrhenius plot is a common feature for many enzymes [1]. Although there are in principle several possible explanations for this non-linearity [2] there are many indications that fluidity changes in the phospholipid bilayer are involved. This is particularly well documented for ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) [3].

The relation between phase-transition temperature of the phospholipids and the break-point temperature for the enzyme activity is not very clear since

there is generally no agreement between the two values [4,5]. To explain these discrepancies, the existence of a lipid annulus with tightly bound phospholipids differing in properties from the bulk lipids, has been suggested. For a review see [6].

The experimental evidence for annular lipid is derived mainly from ESR work [7,8]. However, the precise nature of the immobilized boundary lipid is not well-defined.

On the basis of NMR data, Brown et al. [9] have concluded that translational exchange of annular lipids should be seriously considered as an alternative to the immobilized boundary lipid model. We have investigated the influence of parameters, that can shift phase-transition temperatures of lipids, on the break-point in the Arrhenius plot of $(Na^+ + K^+)$-ATPase.

As chemical parameters we used compounds (benzylalcohol and desipramine) which fluidize lipid bilayers, and shift the transition temperature to a lower value [8,10]. As a thermodynamic parameter we used hydrostatic pressure, which shifts the transition temperature of phospholipids to a higher value by about 21 K/1000 atm as shown previously [5,11]. The influence of these parameters on the break-point for the $(Na^+ + K^+)$-ATPase is discussed in terms of a lipid annulus around the protein *.

Materials and Methods

Enzyme preparation. $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was obtained from pig kidney outer medulla according to Jørgensen [12] with minor modifications. The microsomal fraction was incubated with sodium dodecyl sulfate (SDS) (SDS 0.56 mg/ml; ATP 3 mM; imidazole 25 mM (pH 7.5); EDTA 2 mM; protein concentration 1.4 mg/ml).

After incubation for 30 min at room temperature the separation of the $(Na^+ + K^+)$-ATPase is obtained by centrifugation through a linear sucrose gradient (10—35% sucrose (by weight)).

After centrifugation for 120 min at 60 000 rev./min in a Beckmann Ti60 fixed-angle rotor, the $(Na^+ + K^+)$-ATPase is obtained as a band at 28—30% sucrose (by weight).

The specific activity is 1200—1500 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ and 99.5% is ouabain sensitive.

Some of the experiments were done using a less purified preparation which was obtained after incubation of the microsomal fraction with deoxycholate and separation by differential sedimentation. The final activity was 400—600 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ and 95% is ouabain sensitive. Except for the lower specific activity of this deoxycholate preparation, there was no significant difference between the results obtained with the two types of preparations.

The preparations were stored at 0°C. The purer (SDS) preparation was stable for several weeks, the deoxycholate-treated preparation decreased in activity about 10% in two weeks.

Chemicals. Desipramine hydrochloride was obtained from Ciba-Geigy NV, NADH and phospho*enol*pyruvate diTris salt, were from Sigma. Pyruvate kinase,

* Some of these results appeared as an abstract [36].

lactate dehydrogenase and disodium ATP were from Boehringer. The sodium salt of the ATP was converted to the Tris salt by ion exchange on a Biorad AG50W × 8 cation-exchange resin [13].

Determination of enzyme activity. The coupled enzyme system as described by Barnett [14] was used. The reaction mixture contained: Na^+ 130 mM; K^+ 20 mM; Mg^{2+} 3 mM; Tris/ATP 3 mM; Tris buffer 30 mM (pH 7.4); lactate dehydrogenase 35 units/ml; pyruvate kinase 40 units/ml; phospho*enol*pyruvate 1.5 mM; NADH 0.2 mg/ml.

The reaction was started after addition of the enzyme to a final concentration of 1—5 μg/ml and was followed in an Aminco DW-II spectrometer.

The ouabain-insensitive part (detectable only in the deoxycholate-treated preparation) was measured in the same way but in the presence of 1 mM ouabain and with an enzyme concentration, ten times higher.

The K^+-stimulated *p*-nitrophenyl phosphatase activity was measured [15] as the amount of *p*-nitrophenol produced in a solution containing: *p*-nitrophenyl phosphate 10 mM, Mg^{2+} 10 mM; Tris buffer 30 mM (pH 7.4) and K^+ 50 mM and a protein concentration of 4 μg/ml. As a blank KCl was substituted by choline chloride (50 mM). According to Skou [16] these values represent optimum conditions for maximal activity. The reaction was stopped by addition of 100 μl trichloroacetic acid (50%) to 1 ml reaction solution and the color developed by addition of 2 ml Tris/base (0.5 M) (to 1 ml reaction solution). The color was measured at 410 nm in a Carry 118 spectrophotometer.

Protein was determined according to Lowry et al. [17] with bovine serum albumin as a standard.

Activity measurement under pressure. For the ATPase reaction, the coupled enzyme system as described above was used. The reaction solution was transferred into a spectrophotometer cell and covered with a rubber membrane. The cell was placed in a thermostatically controlled high pressure vessel which was filled with oil. This procedure takes 3 min; after another 5 min needed for temperature equilibration, the reaction was followed as the decrease in absorbance at 340 nm with a Zeiss PMQ II photometer. The ouabain-insensitive ATPase activity was pressure independent and was substracted from the total ATPase activity to obtain the (Na^+ + K^+)-stimulated ATPase.

It was possible to measure the activity at different pressures from 1 atm up to 600 atm and back to 1 atm in one single run. The pressure effect was reversible for pressure values below 700 atm. For the *p*-nitrophenyl phosphatase activity, 1 ml of the reaction mixture was incubated in a syringe, a convenient pressure-transmitting device. The total incubation time in the high pressure vessel was 20 min. 5 min were needed for the application and release of the pressure. A correction was made for the amount of *p*-nitrophenol produced in this 5 min period. A blank was run at the same time. The effects of pressure and temperature on the pH due to the change of the p*K* of the Tris buffer, are —0.3 pH units for a ΔT of +10°C and —0.02 pH unit for a ΔP of +1000 atm. These pH shifts have no influence on the activity and can be neglected.

Results

Pressure dependence of the ($Na^+ + K^+$)-ATPase activity

The ($Na^+ + K^+$)-ATPase activity decreases with increasing pressure. According to the relation,

$$\frac{d \ln v}{dP} = -\frac{\Delta V^{\#}}{RT}$$

a plot of $\ln v$ against P should give a straight line with a slope of $-\Delta V^{\#}/RT$ ($\Delta V^{\#}$ is the activation volume in $ml \cdot mol^{-1}$ and R is the gas constant: $0.082\ l \cdot atm \cdot deg^{-1} \cdot mol^{-1}$).

As indicated in Fig. 1 we obtain a plot with a discontinuity in the slope at 240 atm (25.2°C). From the slopes above and below the breakpoint, two $\Delta V^{\#}$ values are obtained.

A similar behavior is observed in the temperature dependence study: in the Arrhenius plot for this enzyme there is also a breakpoint and two values for the activation energy. The activation volumes, and the position of the breakpoint are independent of the ATP concentration. This is illustrated by the position of the filled symbols in Fig. 1. In independent control experiments we observed that the activity for pressure values between 1 and 600 atm, is independent of the ATP (Mg^{2+}) concentration in the range from 2 to 6 mM.

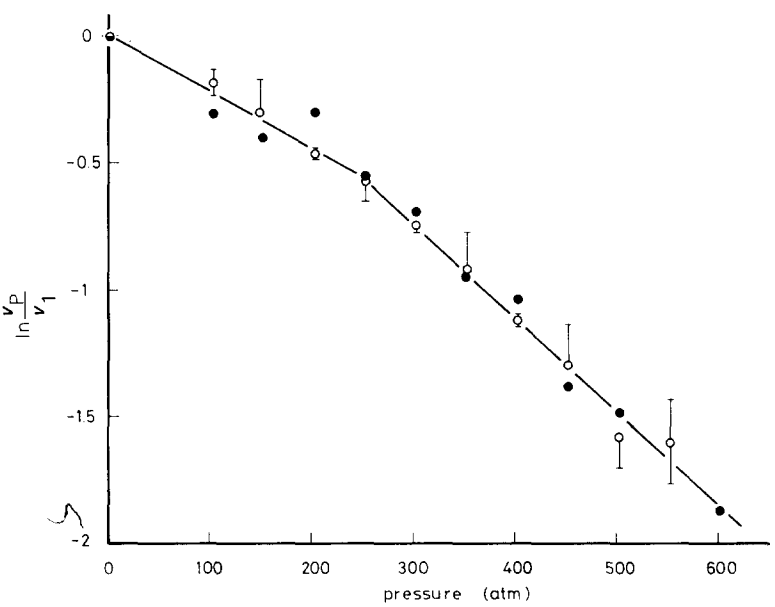

Fig. 1. Pressure dependence of the ($Na^+ + K^+$)-ATPase activity. The natural logarithm of the ratio v_P/v_1 is plotted against pressure: v_P and v_1 are the enzyme activities at a pressure P and 1 atm, respectively. Conditions: temperature 25.2°C; Na^+ 130 mM; K^+ 20 mM; Mg^{2+} 3 mM; ATP (Tris salt) 3 mM, and Tris buffer 30 mM (pH 7.4). The coupled enzyme system was used as described in th text. The open symbols are the mean values of three determinations (error bars indicate S.E.). The closed symbols represent an experiment in the same conditions but with 5 mM ATP and 5 mM Mg^{2+}. The curve is the best fit for the open symbols only.

TABLE I

BREAK-POINT PRESSURE AND ACTIVATION VOLUMES FOR $(Na^+ + K^+)$-ATPase AS A FUNCTION OF TEMPERATURE

The activity was measured continuously by the coupled enzyme method as described in the text. The conditions were as indicated in Fig. 1 (open symbols). In a single experiment the pressure was changed in discrete steps from 1 atm to 600 atm and back to 1 atm. This was repeated several times to obtain a plot as shown in Fig. 1. The data are mean values for a number of such plots (as indicated). These different determinations were mostly with different enzyme preparations.

Temperature (°C)	No. of determination	Break-point pressure P_b (atm ± S.E.)	$\Delta V^{\#}$ $(P < P_b)$ (ml/mol)	$\Delta V^{\#}$ $(P > P_b)$ (ml/mol)
20.7	3	85 ± 35	*	94
22.8	1	150	*	88
23.5	1	185	53	83
25.2	3	240 ± 5	55	90
27.1	2	295 ± 14	53	104
29.5	7	400 ± 34	59	**

* The break-point pressure is close to 1 atm, so that an accurate estimation of the slope for $P < P_b$ is not possible.

** The break-point pressure is close to the upper limit (600 atm) which could be obtained without deactivation of the enzyme so that an accurate estimation of the slope for $P > P_b$ is not possible.

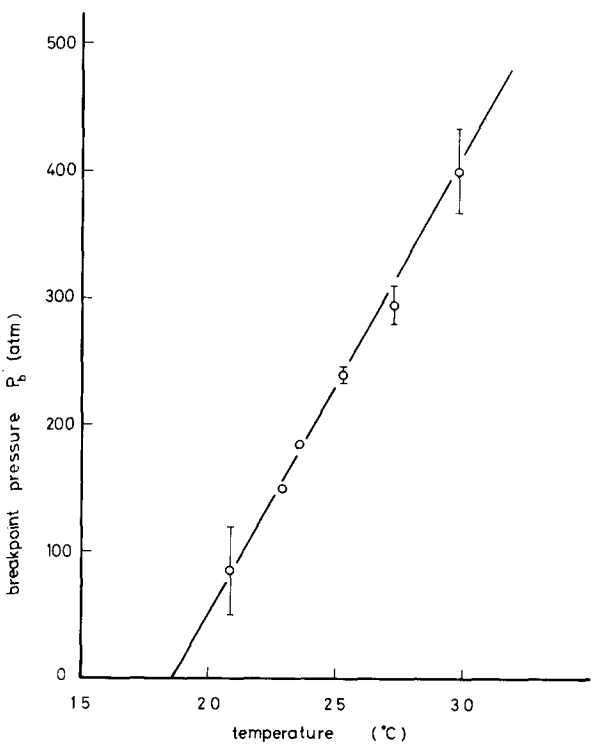

Fig. 2. Temperature dependence of the breakpoint pressure (P_b). The values for P_b at different temperatures were determined from plots such as in Fig. 1. The points are mean values for a number of determinations. (indicated in Table I). The error bars represent S.E. The experimental conditions were as described under Fig. 1 (open symbols).

The experiment, illustrated in Fig. 1 was repeated at different temperatures with the results summarized in Table I. The position of the breakpoint is temperature dependent. It shifts to higher pressure with increasing temperature. The values for the activation volume both above and below the breakpoint pressure, are not changed significantly by a change in temperature. The mean values and standard error for a number of experiments between 20°C and 30°C are $\Delta V^{\#}$ $(P < P_b)$ = 56.6 ± 2.6 ml/mol (mean of 13 experiments) and $\Delta V^{\#}$ $(P > P_b)$ = 93 ± 6.5 ml/mol (mean of ten experiments).

In Fig. 2 the shift of the breakpoint pressure P_b with temperature is plotted. The increase of P_b is linear with a slope of 36.1 ± 0.5 (S.D.) atm · deg^{-1}. This plot shows the relation between the temperature (T_b) and the pressure (P_b) at the breakpoint. The temperature corresponding to a P_b value of 1 atm is in fact the value for the breakpoint temperature in the Arrhenius plot (measured at 1 atm). From Fig. 2 we obtain a value of 18.5°C for T_b at 1 atm. The value obtained from the Arrhenius plot is about 21°C [15].

Although the two values are not identical the agreement is good, since there is a large error on the determination of a breakpoint pressure in the range of 1—100 atm.

The dT/dP value which will be used in the discussion is the inverse of the value obtained from the slope in Fig. 2.

$$\frac{dT}{dP} = 27.7 \pm 0.4(\text{S.D.})\ \text{K/1000 atm}$$

Pressure dependence of the p-nitrophenyl phosphatase activity

The *p*-nitrophenyl phosphatase activity decreases with increasing pressure (Fig. 3). In contrast to the ATPase activity, there is no breakpoint in this plot.

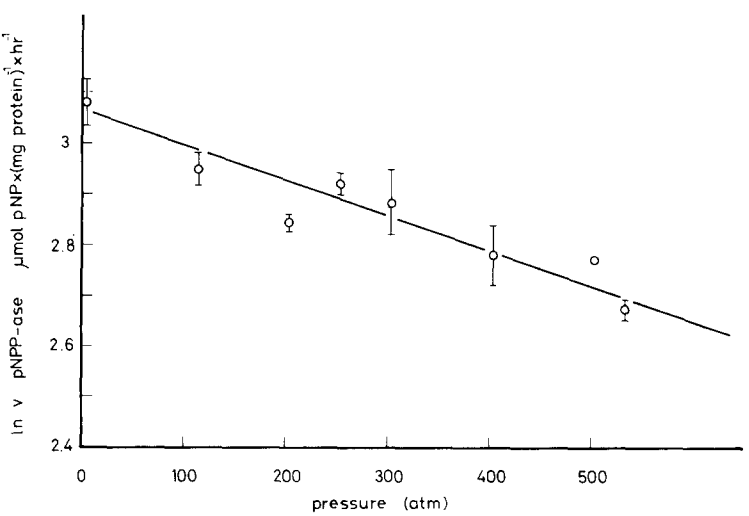

Fig. 3. Pressure dependence of the *p*-nitrophenyl phosphatase activity. The natural logarithm of the *p*-nitrophenyl phosphatase activity is plotted as a function of pressure. Conditions: temperature 25.2°C; *p*-nitrophenyl phosphate 10 mM, Mg^{2+} 10 mM, K^+ 50 mM; Tris buffer 30 mM (pH 7.4). The symbols are the mean values for three determinations. The error bars indicate S.E. p-NP = *p*-nitrophenol; p-NPP, *p*-nitrophenyl phosphate.

The activation volume is positive but is much smaller than for the ATPase reaction. From the slope of the plot in Fig. 3 we obtain $\Delta V^{\#} = 17 \pm 1$ (S.D.) ml/mol. We find that the Arrhenius plot for the *p*-nitrophenyl phosphatase activity in the conditions used here is also linear (data not shown) with an activation energy of 11 kcal/mol. This is in agreement with other observations [15].

Effects of desipramine and benzylalcohol on the Arrhenius plot of ($Na^+ + K^+$)-ATPase

The Arrhenius plot for ($Na^+ + K^+$)-ATPase is non-linear. There is a breakpoint at about 21°C. The activation energy is about twice higher at temperatures below this breakpoint [15].

We have examined the effect of lipophilic drugs on the Arrhenius plot of this enzyme.

In one experiment 0.5 mM desipramine was used. A second experiment was in the presence of 25 mM benzylalcohol.

The concentration of the drugs used is limited due to their inhibition effect on the enzyme activity.

We could not observe a significant change in the position of the inflection point in either of the two cases. The only effect of the drugs is a change in the activation energy due to the fact that the inhibition is temperature dependent.

TABLE II

EFFECT OF PRESSURE ON PHASE TRANSITIONS IN PROTEINS, PHOSPHOLIPIDS AND ALCANES

	dT/dP (K/1000 atm)	Transition temperature at 1 atm (°C)	Ref.
Protein conformational changes			
Ribonuclease	2.2		19
Chymotrypsinogen	1.7		20
Metmyoglobin	6.5		21
poly(benzyl-L-glutamate)	5.6		22
Phospholipid dispersions			5
Dilauroyl phosphatidylcholine	17	0.5	
Dimyristoyl phosphatidylcholine (DMPC)	20.5	24	
Dipalmitoyl phosphatidylcholine	21.8	41.5	
Dilauroyl phosphatidyl ethanolamine	21.5	31	
DMPC + Na^+-tetraphenylborate [a]	20.5	14.9	
DMPC + chlorpromazine [b]	21	21	
DMPC + cholesterol [c]	20	23	
Alcanes			
n-C-18	25.7 (1 atm) 21 (1000 atm)		23
Polyethylene (extended chain)	26 (1 atm) 24 (1000 atm)		24
Break-point in Arrhenius plots			
A. nitrogenase	20	22 [d]	5
($Na^+ + K^+$)-ATPase	27.7	20.5 [d]	this work

[a] Na^+-tetraphenylborate (10^{-3} M).
[b] Chlorpromazine ($1.25 \cdot 10^{-4}$ M) unbuffered solution at pH 6.
[c] Cholesterol (20 mol%).
[d] Temperature of the breakpoint in the Arrhenius plot (T_b).

Discussion

Pressure effects on phospholipid and protein transitions

In previous work [5,18] we have shown that the transition temperature for pure synthetic phospholipids increases linearly with increasing pressure. The slope of this pressure dependence dT/dP is about 21 K/1000 atm. This value is only slightly dependent on chain length and polar head group. Moreover the dT/dP value is not affected by the presence of lipophilic molecules that shift the transition temperature. As listed in Table II the dT/dP value for a true melting process in *n*-alcanes or polyethylene is not very different from the dT/dP value for phospholipid phase transitions. The physical meaning for dT/dP is given by the Clausius-Clapeyron equation.

$$\frac{dT}{dP} = \frac{T\,\Delta V}{\Delta H}$$

(ΔV and ΔH are the volume change and enthalpy change, for the melting process, respectively).

For lipid phase transitions the ratio $\Delta V/\Delta H$ is only slightly dependent on chain length. For an *n*-alcane melting process, ΔV and ΔH are about three times higher than for a phase transition [25] but the ratio $\Delta V/\Delta H$ is about the same.

Protein conformational changes, in contrast to the chain-melting process in hydrocarbons, have very different values for dT/dP, dependent on the $\Delta V/\Delta H$ ratio for the process involved. In the cases where both the ΔV and the ΔH value are known (Table II) dT/dP is much smaller than for lipid melting processes.

Pressure effect on the enzyme activity

The effect of pressure on the steady-state activity of the ATPase is quite high. The activation volume ($\Delta V^{\#}$ = 56.6 ml/mol cannot be accounted for by a single chemical process [26]. A possible organic model reaction for the hydrolysis of ATP is the hydrolysis of the acetyl phosphate dianion. This reaction has an activation volume of −19 ml/mol [27].

High positive activation volumes were also observed by Penniston [28] for a number of ATPase and ATP-P_i-exchanging enzymes. Penniston attributed the large activation volumes to the presence of subunits in these enzymes. The pressure dissociation of enzymes and proteins is well-documented in several instances [29].

Another possible cause for the high activation volume could be rate-limiting changes in the conformation of the enzyme. In the case of ($Na^+ + K^+$)-ATPase, conformational changes have been proposed as part of the reaction mechanism [30].

The $\Delta V^{\#}$ value for *p*-nitrophenyl phosphatase is much lower than for the ATPase reaction (17 ml/mol). As discussed by Glynn and Karlish [31], the reaction mechanism for the *p*-nitrophenylphosphatase activity involves only a part of the enzyme and is probably not accompanied by ion movements. The conformational changes in the ATPase reaction which occur during the ion translocation are probably not involved in the *p*-nitrophenyl phosphatase reaction, which might explain the small and constant value for $\Delta V^{\#}$ and $\Delta H^{\#}$.

Temperature and pressure effect on the activation parameters

The most important characteristic for this enzyme is that the activation parameters $\Delta V^{\#}$ and $\Delta H^{\#}$ change with pressure and temperature, respectively. The process which induces these changes in activation parameters at the breakpoint, has the thermodynamical characteristics, expressed by the dT/dP value, of a lipid chain-melting process. For the breakpoint in the Arrhenius plot of the $(Na^+ + K^+)$-ATPase we find a pressure dependence (dT/dP) of 27.7 K/1000 atm.

If this value is compared with the values listed in Table II, it can be concluded that it is in the range typical for a lipid chain-melting process and far from the values for protein conformational changes. In our interpretation, this is an indication that the conformational change, occurring at the breakpoint, is accompanied by a melting of aliphatic chains. The same conclusions were drawn for an other enzyme system, the *Azotobacter* nitrogenase, where the same pressure dependence for the breakpoint was found [5].

This is not the case for the *p*-nitrophenyl phosphatase activity. The activation parameters $\Delta V^{\#}$ and $\Delta H^{\#}$ are constant in the range studied. This means that the activation step in the *p*-nitrophenyl phosphatase is not affected by the lipid-melting process which affects the ATPase reaction. Similar conclusions about the Arrhenius plot for the *p*-nitrophenyl phosphatase activity were drawn by Walker and Wheeler [15].

Pressure versus drug effect

The simplest interpretation of the observed effects is the assumption of the existence of a lipid annulus. The aliphatic chains in the lipid-protein complex can have a much lower fluidity than the chains in the bulk lipids, for sterical reasons.

The pressure effect on the breakpoint (dT/dP value) suggests that an aliphatic chain-melting phenomenon in the annular region takes part in the conformational changes of the $(Na^+ + K^+)$-ATPase.

The pressure experiments cannot discriminate between a lipid phase transition in the lipid annulus and a phase separation in the bulk lipids. The dT/dP value for a phase separation has not been measured yet, but it could be comparable to the value for phase transitions.

The annular lipid hypothesis, however, offers a more convenient explanation for experiments with lipophilic drugs.

Hesketh et al. [8] reported that 50 mM benzylalcohol induces a shift in the breakpoint in the Arrhenius plot for Ca^{2+}-ATPase (reconstituted with dipalmitoyl phosphatidylcholine).

We do not observe the same phenomenon in our $(Na^+ + K^+)$-ATPase preparation. Our interpretation is that the lipophilic molecules fail to interact with the lipid annulus, the interaction being dependent on the relative affinity of the drug and lipid molecules for the hydrophobic sites on the protein.

The value of this argument, which is favor of the lipid annulus hypothesis, is only limited by the fact that a quantitative knowledge of the interaction of the drugs with unsaturated lipids is not available. The hydrophobic sites of the protein must be occupied in order to restore the activity. This can be achieved by lipids and in certain cases also by detergents. In a recent study with Ca^{2+}-ATPase Dean and Tanford [32] have shown that the breakpoint in the

Arrhenius plot for this enzyme can be observed even after almost complete delipidisation and substitution of the lipids with a detergent.

It would be very interesting to show if the breakpoint in this case has the same dT/dP shift as for the $(Na^+ + K^+)$-ATPase in our study. If the conformational change, responsible for the change in activation parameters at the breakpoint, is induced by fluidity changes in aliphatic chains, a similar dT/dP value should be expected.

If the breakpoint in the Arrhenius plot and the log activity versus pressure plot reflect melting processes of hydrocarbons, interesting conclusions can be drawn from the changes in slope at high pressure or low temperatures. Since the ATPase activity of the enzyme decreases with increasing pressure, this reflects the fact that ordered hydrocarbons are inhibitory. This was also observed for nitrogenase by us [5] and by Pequeux and Gilles [33] for the membrane ATPase from various sources. We have found only one example in the literature, the phosphate transport in erythrocytes, where the slope of the pressure curve bents upwards, i.e. the transport increases by the ordered phase of the phospholipids [34]. This demonstrates that pressure is a useful tool in testing some hypotheses made in connection with the molecular mechanism of local anesthetics [35].

Acknowledgements

The authors are very grateful to Professor L. De Maeyer for his encouragement and support. This work has been supported by grant No. 20487 of the F.G.W.O. and grant No. 76/81-II 6 'geconcerteerde onderzoeksactie', and by the Belgian National Science Foundation (N.F.W.O.).

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