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Temperature-dependencies of various catalytic activities of membrane-bound Na^+/K^+-ATPase from ox brain, ox kidney and shark rectal gland and of $C_{12}E_8$-solubilized shark Na^+/K^+-ATPase

Mikael Esmann and Jens Christian Skou

Institute of Biophysics, University of Aarhus (Denmark)

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The temperature dependence of ouabain-sensitive ATPase and phosphatase activities of membrane fragments containing the Na^+/K^+-ATPase were investigated in tissue from ox kidney, ox brain and from shark rectal glands. The shark enzyme was also tested in solubilized form. Arrhenius plots of the Na^+/K^+-ATPase activity seem to be linear up to about 20°C, and non-linear above this temperature. The Arrhenius plots of mammalian enzyme (ox brain and kidney) were steeper, especially at temperatures below 20–30°C, than that of shark enzyme. The Na^+-ATPase activity showed a weaker temperature-dependence than the Na^+/K^+-ATPase activity. The phosphatase reactions measured, K^+-stimulated, Na^+/K^+-stimulated and Na^+/K^+/ATP-stimulated, also showed a weaker temperature-dependence than the overall Na^+/K^+-ATPase activity. Among the phosphatase reactions, the largest change in slope of the Arrhenius plot was observed with the Na^+/K^+/ATP)-stimulated phosphatase reaction. The Arrhenius plots of the partial reactions were all non-linear. Solubilization of shark enzyme in $C_{12}E_8$ did not change the curvature of Arrhenius plots of the Na^+/K^+-ATPase activity or the K^+-phosphatase activity. Since solubilization involves a disruption of the membrane and an 80% delipidation, the observed curvature of the Arrhenius plot can not be attributed to a property of the membrane as such.

Introduction

Experiments designed for the elucidation of the kinetic model for the enzymatic and transport activity of Na^+/K^+-ATPase are performed at temperatures which range from 0 to 37°C. There are several reports of the effect of temperature on the overall Na^+/K^+-ATPase activity and on the activities associated with partial reactions such as Na^+-ATPase and the phosphatase reactions [1–14]. Most of these experiments are performed on enzymes from a single source.

For a comparison of transient kinetic studies we needed to know the effect of temperature on the activities of enzyme from mammalian kidney and brain (ox) and from rectal glands of shark (*Squalus acanthias*), in membranous and solubilized form, measured under identical conditions. These three tissues are frequently used for pre-

Abbreviations: $C_{12}E_8$, octaethyleneglycoldodecylmonoether; SDS, sodium dodecyl sulphate; α-subunit, the 112 kDa catalytic subunit; β-subunit the 35 kDa glycoprotein; CDTA, *trans*-1,2-cyclohexenylenedinitrilotetraacetic acid; DOC, deoxycholate; pNPPase, phosphatase reaction associated with the ouabain-sensitive Na^+/K^+-ATPase; pNPP, *p*-nitrophenyl phosphate; PEP, phospho*enol*pyruvate.

Correspondence: M. Esmann, Institute of Biophysics, Ole Worms Alle 185, University of Aarhus, DK-8000 Aarhus C, Denmark.

paration of enzyme, and a comparison of the effect of temperature on the enzymatic activity therefore seemed appropriate.

The results show that a plot of the natural logarithm of the activity versus the reciprocal absolute temperature (an Arrhenius plot) has no obvious breakpoints (i.e., does not consist of two linear sections), which is in contrast to previous reports. It is also found that a 80% delipidation through solubilization does not alter the shape of the Arrhenius plot, indicating that the temperature-dependent changes in activity are not due to the membrane structure as such.

The results obtained are trivial in the sense that they do not add to the understanding of the kinetics of the enzyme reaction. However, they may be useful for other workers in the field for interpretation of kinetic experiments, and are therefore reported.

Materials and Methods

Preparation of enzyme from ox kidney and brain and from shark rectal gland

Ox kidney enzyme is prepared using a modification of the SDS procedure [15]. Kidney microsomes are treated with SDS in the presence of 3 mM ATP for 30 min at 23°C, and subjected to differential centrifugation as described for the shark enzyme [16]. The specific Na^+/K^+-ATPase activity of the membrane-bound kidney enzyme is about 800 μmol/mg protein per h (see Table I) with a purity of about 30–40%.

Ox brain enzyme is prepared using the DOC method and NaSCN to inhibit ouabain-insensitive Mg^{2+}-ATPase activity [17]. The specific Na^+/K^+-ATPase activity is usually about 300 μmol/mg protein per h (see Table I), and the purity is estimated to be 10–15%.

Shark rectal gland enzyme is purified with DOC, as previously described from rectal glands of the spiny dogfish (*S. acanthias*), omitting saponin in the preparative procedure [16]. The specific Na^+/K^+-ATPase activity of the membrane-bound enzyme is between 1100 and 1500 μmol/mg per h (see Table I), and the purity – judged from the content of α- and β-subunits – is 50–70%.

Procedure for solubilization of shark enzyme in $C_{12}E_8$

The enzyme is solubilized at 4°C using $C_{12}E_8$ as follows. To 1 vol. of enzyme (4 mg protein/ml in 20 mM histidine/25% glycerol (pH 7.0)) is added 1 vol. $C_{12}E_8$ (8 mg/ml) in 30 mM histidine (pH 7.0), and the suspension is vigorously stirred. At this detergent/protein ratio all Na^+/K^+-ATPase protein is solubilized [18]. The suspension is referred to as solubilized shark enzyme.

Definition and measurement of catalytic activities

Na^+/K^+-ATPase activity is defined as the ouabain-sensitive ATP hydrolysis in the presence of 130 mM NaCl/20 mM KCl/4 mM $MgCl_2$/3 mM ATP. The ouabain concentration is 1 mM.

Na^+-ATPase activity is the ouabain-sensitive ATP hydrolysis in the presence of 150 mM NaCl/4 mM $MgCl_2$/3 mM ATP.

K^+-pNPPase activity is the K^+-stimulated phosphatase activity associated with the enzyme. It is measured in the presence of 150 mM KCl/20 mM $MgCl_2$/10 mM pNPP. There is no detectable phosphatase reaction in the absence of KCl, so a KCl blank is used in these experiments. (Ouabain inhibition is too slow to be of practical use as a blank for measurement of the phosphatase reaction, but it can be shown that all K^+-pNPPase activity is ouabain-inhibitable.)

Na^+/K^+-pNPPase activity is the small increase in phosphatase activity seen with low KCl concentrations in the presence of 140 mM NaCl. It is

TABLE I

SPECIFIC ACTIVITIES OF OVERALL Na^+/K^+-ATPase HYDROLYSIS AND OF PARTIAL REACTIONS OF OX BRAIN AND KIDNEY ENZYME AND OF SHARK ENZYME AT 40°C.

Values for hydrolysis are given in μmol P_i liberated/mg protein per h, measured as given in the Materials and Methods Section.

Activity	Brain	Kidney	Shark rectal gland
Na^+/K^+-ATPase	321	830	1110
Na^+-ATPase	–	–	36
K^+-pNPPase	42	151	187
Na^+/K^+-pNPPase	6.5	–	20
Na^+/K^+/ATP-pNPPase	28.3	–	105

measured in 10 mM KCl/140 mM NaCl/20 mM $MgCl_2$/10 mM pNPP.

Na^+/K^+/ATP-pNPPase activity is the further increase in phosphatase activity seen when ATP is added in the presence of low KCl and high NaCl concentration. The pNPP hydrolysis is measured in the presence of 10 mM KCl/140 mM NaCl/20 mM $MgCl_2$/10 mM pNPP/0.1 mM ATP. In addition, the test solution contained 10 mM PEP and 1 μg pyruvate kinase/ml in order to keep the ATP concentration at 0.1 mM.

Values for activities are given in per cent of the activity at 40 °C to ease comparisons of the different Arrhenius plots. The absolute values for the activity at 40 °C is shown in Table I for all activities reported here.

The hydrolysis time at a given temperature is adjusted so that no more than 10% of the substrate is hydrolysed during incubation. In some cases the PEP/pyruvate kinase ATP-generating system is also used. Hydrolysis of ATP or pNPP is linear with time, at least up to 5 min. At temperatures around 40 °C, initial values for the hydrolysis are used due to the thermal inactivation of the enzyme.

ATP hydrolysis is estimated from the liberation of inorganic phosphate using the Fiske-SubbaRow reagent, and pNPP hydrolysis from the increase in absorbance at 410 nm due to formation of *p*-nitrophenol.

Albumin is included in the Na^+/K^+-ATPase test solution for measurement of shark Na^+/K^+-ATPase activity. Albumin (10–20 μg/ml) increases the specific Na^+/K^+-ATPase activity of shark enzyme by 20% [16], but has no effect on ox kidney or brain enzyme. The shape of the Arrhenius plot is not altered by albumin (not shown).

Protein concentration is estimated using the procedure of Lowry et al. [19], with albumin as standard. Albumin concentration is estimated from the absorbance at 280 nm, with 1 mg/ml giving an absorbance of 0.67.

$C_{12}E_8$ is obtained from Nikko Chemicals (Tokyo) and albumin from Behringwerke.

Results

The temperature-dependence of the membrane-bound Na^+/K^+-ATPase activity is influenced by the proton concentration at which the measurement is carried out. The activity measured at 10, 20 and 37 °C in the pH range 6.5 to 8.2 shows that at pH 7.2–7.8 there is a broad optimum at all temperatures, whereas there is a decrease in activity below pH 7 and above pH 8 (Fig. 1). Since most buffers change p*K* with temperature, all measurements that follow are performed at optimum pH at the given temperature. A pH of 7.5, adjusted at the given temperature, proved to be sufficient, in agreement with observations of Walker and Wheeler [12]. Note that Na^+/K^+-ATPase and K^+-pNPPase activities of $C_{12}E_8$-solubilized enzyme are measured at pH 7.0 (cf. Fig. 6), since there is a downwards shift in pH-optimum upon solubilization [18].

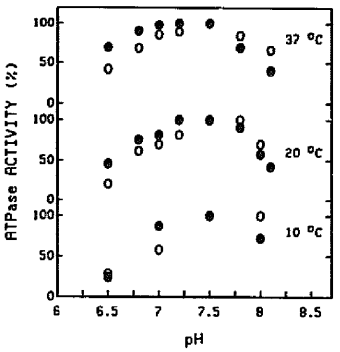

Fig. 1. pH Optimum for shark and ox kidney Na^+/K^+-ATPase activities at various temperatures. The Na^+/K^+-dependent ATP hydrolysis was measured at a range of pH values from 6.5 to 8.1 to establish the pH optimum for measurement of Na^+/K^+-ATPase activity. pH is adjusted at the temperatures indicated, and the activity is measured for shark (●) and ox kidney enzymes (○). Values for each set of data are given in % of optimum at 10, 20 and 37 °C, respectively.

Fig. 2 shows the variation with temperature of Na^+/K^+-ATPase activity of enzyme from ox brain, from ox kidney and from rectal glands of shark (*S. acanthias*) scaled to 100% at 40 °C. The two mammalian enzymes have nearly the same temperature-dependence, but the activity at a given temperature (relative to that at 40 °C) is consistently higher for shark enzyme.

Fig. 3A shows a replot of the data from Fig. 2, with the natural logarithm of the activity on the ordinate and a reciprocal absolute temperature on the abscissa (an Arrhenius plot). Also shown is the

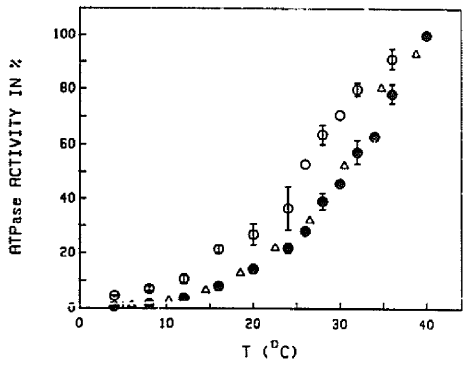

Fig. 2. Na^+/K^+-ATPase activity of ox brain, ox kidney and shark rectal gland enzyme as a function of temperature. Na^+/K^+-ATPase activity is measured as given in Materials and Methods and is expressed as per cent of the activity obtained at 40°C. Data shown are for shark enzyme (○), ox kidney (●) and ox brain (△). The error bars indicate S.D. for 3–5 experiments.

Na^+-ATPase activity of shark enzyme. Fig. 3B shows data for Na^+/K^+-ATPase activity from four different experiments with shark enzyme. The curves in Fig. 3A all show a smoothly changing slope throughout the high temperature range (20–40°C). The slope of the Na^+/K^+-ATPase activity curve does, however, tend to approach a constant value below 16–20°C, corresponding to an activation energy of about 21 kcal/mol (cf. Fig. 3B).

The slope of the curve for Na^+/K^+-ATPase activity of the shark enzyme is smaller throughout the temperature range than that of the mammalian enzymes (Fig. 3A). At low temperatures, the estimated activation energies of the mammalian enzymes are in the range 28–30 kcal/mol, considerably higher than the value of 21 kcal/mol obtained for shark enzyme. The slope of the curve for the Na^+-ATPase activity of the shark enzyme is lower than that of the Na^+/K^+-ATPase activity, about 15 kcal/mol versus 21 kcal/mol (cf. Figs. 3A and 3B).

The finding that the Arrhenius plot does not consist of two intersecting straight lines, but rather of a linear portion (at lower temperatures) followed by a smoothly changing curve at higher temperatures, is in contrast to findings reported in the literature [1–14]. The linearity at low temperatures may suggest a breakpoint at around 20°C. The activation energies given here are obtained from a linear regression analysis of the data points in the range where the straight line is drawn. No further statistical analysis has been made.

Fig. 4 shows the pNPPase activities of shark enzyme. In the high temperature range a change in

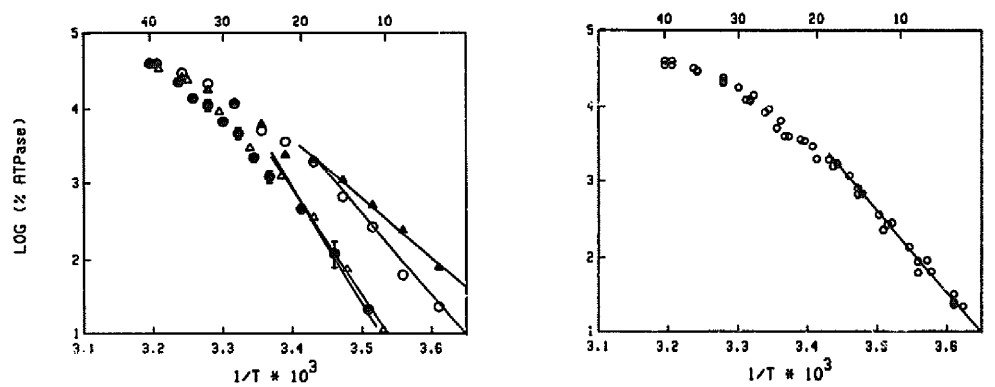

Fig. 3. Arrhenius plots of Na^+/K^+-ATPase activity of ox brain, ox kidney and Na^+- and Na^+/K^+-ATPase activity of shark enzyme. (A) Data from Fig. 2 replotted with the natural logarithm of the activity on the ordinate and the reciprocal absolute temperature on the abscissa. Symbols are as in Fig. 2, with the addition of Na^+-ATPase activity for shark enzyme (▲). Straight lines indicate activation energies in the low temperature range for shark Na^+/K^+-ATPase (21.4 kcal/mol, ○), shark Na^+-ATPase (15.4 kcal/mol, ▲), kidney Na^+/K^+-ATPase (30.4 kcal/mol, ●) and brain Na^+/K^+-ATPase (27.8 kcal/mol, △). (B) Several sets of data of the Na^+/K^+-ATPase activity of shark Na^+/K^+-ATPase, replotted with the natural logarithm of the activity on the ordinate and the reciprocal absolute temperature on the abscissa. The straight line indicates a slope of 21.4 kcal/mol.

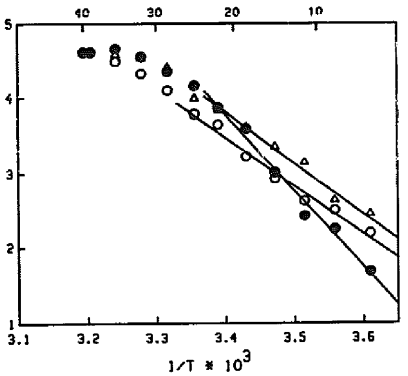

Fig. 4. Arrhenius plots of phosphatase activities of shark enzyme. The three activities associated with the phosphatase reaction of the shark enzyme are shown. Symbols indicate the activities of K^+-pNPPase (open circles), the Na^+/K^+-pNPPase (△) and the Na^+/K^+/ATP-pNPPase (●). The linear portions of the low-temperature part of the curves, indicated by straight lines, are equivalent to activation energies of 12.8, 13.5 and 20.1 kcal/mol, respectively.

slope is observed, whereas a linear relationship between the natural logarithm of the activity and reciprocal temperature is approached in the low temperature range. The change in slope is largest

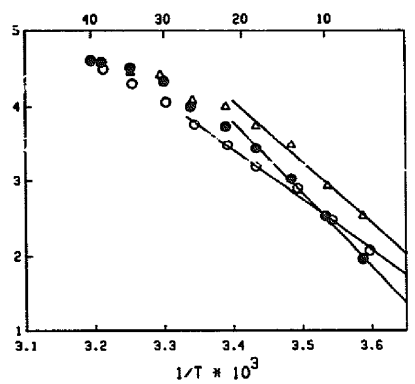

Fig. 5. Arrhenius plots of phosphatase activities of ox brain enzyme. The three activities associated with the phosphatase reaction of the ox brain enzyme are shown. Symbols indicate the K^+-pNPPase (○), the Na^+/K^+-pNPPase (△), and the Na^+/K^+/ATP)-pNPPase (●). The linear portions of the low-temperature part of the curves, indicated by straight lines, are equivalent to activation energies of 13.2, 16.2 and 19.1 kcal/mol, respectively.

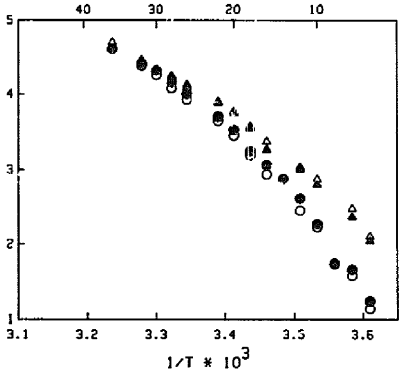

Fig. 6. Comparison of Arrhenius plots of Na^+/K^+-ATPase and K^+-pNPPase activities of the membrane-bound and of the $C_{12}E_8$-solubilized enzyme. This figure shows the effect of solubilization on the Arrhenius plot of the Na^+/K^+-ATPase (○, ●) and K^+-pNPPase activity (△, ▲) of the membrane-bound (○, △) and $C_{12}E_8$-solubilized enzyme (●, ▲).

for the Na^+/K^+/ATP-pNPPase activity, the linear portion of the curve having a slope of approx. 20 kcal/mol. The K^+- and Na^+/K^+-pNPPase activities have about the same slope between 4 and 25°C, with activation energies of about 13 kcal/mol (Fig. 4).

The pNPPase activities of mammalian enzyme (ox brain) are shown in Fig. 5. There is no indication of two linear sections in these plots, but rather a smooth change in slope at high temperatures, as is also seen for the mammalian Na^+/K^+-ATPase activity (cf. Fig. 3). The linear portions of the curve is indicated in the figure, with activation energies in the range 13–19 kcal/mol (Fig. 5).

The effect of solubilization on the shape of the Arrhenius plot of Na^+/K^+-ATPase, or K^+-pNPPase, activity of shark enzyme is shown in Fig. 6. There is no difference between the values obtained with the membrane-bound enzyme and that of the enzyme solubilized in $C_{12}E_8$, indicating that solubilization does not change the slope of the Arrhenius plot.

Discussion

Previous experiments on the effect of temperature on the activity of the Na^+/K^+-ATPase from

kidney and from brain have led to the conclusion that there are breakpoints in Arrhenius plots [1–14]. In the present experiments the curves do not consist of two linear intersecting sections. Provided that pH is optimal and adjusted at the temperature of the activity measurement, the Arrhenius plots give curves with a slope that changes smoothly at higher temperatures but tends to become linear at lower temperatures. The native membrane contains 30–50 mol% cholesterol [22], which will obscure any phase transition in the bilayer. Accordingly, no breakpoints were observed in the temperature dependence of the order parameter deduced from spin-labelled phospholipids added to the Na^+/K^+-ATPase membranes [25]. The smooth change in the slope of the Arrhenius plot is thus in good agreement with the physical properties of the membrane, if it is assumed that breakpoints are to be attributed to properties of the lipid bilayer in the membrane.

The effect of temperature on the activity of kidney and brain enzyme – for example a 6-fold decrease of specific activity of Na^+/K^+-ATPase going from 37°C to 20°C (Table II) – is in agreement with a number of previous reports [3,9,14], but seems to be less pronounced than other values reported [20,21].

In addition, the Arrhenius plot of the Na^+-ATPase and of the pNPPase activities show a curved line, but with a slope which, for the Na^+-ATPase and the K^+-pNPPase activity of all three enzymes, is lower than for the Na^+/K^+-ATPase activities. This is in contrast to experiments by others, who found that the Arrhenius plot of these activities is linear for brain and kidney enzyme [5,12].

The Na^+/K^+/ATP-pNPPase activity in the present experiments has a slope in the Arrhenius plot which comes closer to the Na^+/K^+-ATPase activity than to the K^+-pNPPase activity. Previous experiments on kidney enzyme have given results which show that the Na^+/K^+/ATP-pNPPase activity is linear in the Arrhenius plot [5,12], while in the present experiments it is non-linear, having a shape like the Arrhenius plot of the Na^+/K^+-ATPase activity. We have at present no explanation for this discrepancy, which, however, could be due to the different tissues studied (kidney [5,12]) and bovine brain (the present study).

The effect of delipidation/relipidation experiments on the Arrhenius plot of the Na^+/K^+-ATPase activity suggests that the high energy of activation at temperatures below the transition temperature is due to a lower fluidity of the lipid phase. It seems likely that the lipids in the membrane from the rectal gland of the shark at a given temperature are more fluid than the lipids in the membrane from mammalian tissue, since the shark is adapted to a lower temperature. This may explain why the shape of the curve in the Arrhenius plot is lower for enzyme from shark than from mammalian brain and kidney.

However, solubilization of the shark enzyme does not alter the Arrhenius plot. The solubilized enzyme has only about 50 phospholipid molecules left per enzyme molecule, and about 40 molecules of cholesterol, which is only enough for a single bilayer of lipids around the protein [22]. In this case, there can be no phase transition in the natural lipids but rather a phase transition in $C_{12}E_8$ which has replaced the bulk of the lipids from the membrane. Since there is no difference between the slope of the curve in the Arrhenius plots of the membrane-bound and the solubilized enzyme, this must mean that $C_{12}E_8$ behaves as a natural lipid in the membrane from the point of view of phase transition. On the other hand, $C_{12}E_8$ is homogeneous: if a phase transition determines the change in slope of the Arrhenius plot, one would assume that with $C_{12}E_8$ there would be a distinct breakpoint in the curve.

Another possibility is that it is not a phase transition in the lipids which determines the slope

TABLE II

THE RELATIONSHIP BETWEEN THE Na^+/K^+-ATPase ACTIVITY AT 37°C, 20°C AND 10°C FOR SHARK, BRAIN AND KIDNEY ENZYME

The specific Na^+/K^+-ATPase activity (S.A.) and the ratio between the activities are given at 37°C and 20°C or 10°C, respectively.

	37°C	20°C		10°C	
	S.A.	S.A.	ratio	S.A.	ratio
Shark	1037	320	3.2	108	9.6
Ox brain	271	49	5.6	10.4	26
Ox kidney	701	126	5.6	27	27

of the curves in the Arrhenius plots, but a temperature effect on conformational steps in the protein. Differences in rates of conformational transitions between different species could also explain the different activation energies obtained in the tissues studied here. Little is known about this, except that for the shark enzyme the rate of transition from the E_2 to the E_1 conformation is about 5-times higher than for kidney [23,24], and that the E_2 to E_1 conformational transition is more temperature-sensitive than the reverse reaction [24]. The rate of de-occlusion of Rb^+ from the enzyme is also 5–10-times higher in shark tissue than in kidney tissue [26,27].

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