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ELECTROSTATIC CONTROL BY LIPIDS UPON THE MEMBRANE-BOUND ($Na^+ + K^+$)-ATPase *

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Summary

In this paper, the membrane-bound ($Na^+ + K^+$)-ATPase from bovine brain is shown to be controlled by electrostatic alterations of the charged lipids surrounding the enzyme. The properties under investigation are the enzymatic activity, activation energy and the response of the enzymatic system to temperature. Arrhenius plots of the ATPase activity are biphasic with a break at temperature T_1. The temperature T_1, the activation energies at temperatures above and below T_1, and the enzymatic activity at any constant temperature have been shown to depend upon the concentrations of alkali and alkaline-earth metal ions in the solution. These electrolyte dependencies are ascribed to changes of electrostatic conditions at the lipids surrounding the ATPase.

If the higher electrostatic screening ability of divalent ions is taken into account, the results in the presence of mono- and divalent ions become virtually the same. As a result of this work, it is concluded that electrostatic alterations are transmitted to the ATPase from the lipids of the membrane in which the enzyme is embedded. Inhibition and activation of the enzyme by mono- and divalent metal ions may thus be explained without any auxiliary hypothesis, particularly without postulating specific binding sites for the different ionic species at the protein. In addition, the specific lipid requirement of the ATPase may be understood better in the light of this interpretation.

* Part of this work has recently appeared in abstract form [43].

Introduction

In recent years, it has sometimes been suggested that the functions or transport functions of enzymes could be controlled by membrane lipids [1—3]. This control can be imagined to occur through different properties of the lipid membrane:

(1) A current view is that the state of fluidity of the lipids may regulate the motional freedom of the protein. Thus, it is assumed that in a highly ordered lipid, conformational motions of the enzyme are restricted and vice versa [4—7]. This would become manifest in the enzymatic activity if a conformational change were involved in the rate-determining process of the overall reaction.

(2) Another possibility for enzyme control by membrane lipids is via ionic or electrostatic surface properties. This point is the main concern of this work.

A prerequisite for surface electrostatics to become effective is the presence of lipids with polar head groups in the vicinity of the enzyme. Charged lipid bilayers are known to interact strongly with ions [8—10]. Such an interaction may be purely electrostatic in nature or, particularly with divalent ions, complex formation at the surface may also be involved [11—13].

The interaction of ions with charged membranes, irrespective of the mechanism, in any case influences the surface charge density. The resulting influence upon the surface free energy has been shown to affect the motional parameters of the lipids [14—17], thus enabling the above-mentioned fluidity influence to act on the enzyme.

Moreover, the ionic conditions at the surface, including effects associated with the formation of an electrical double layer, may interfere more directly with the reaction between substrate and enzyme. This is of particular importance when ions participate in the enzymatic reaction.

Membrane-bound ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from bovine brain has been isolated as a particulate membrane preparation without separating the protein from adjacent lipids. This enables the influence of the lipids on the ATP hydrolysis reaction under varying ionic conditions to be followed.

From previous studies with similar ATPase preparations, it is known that the enzymatic reaction is markedly influenced by surrounding lipids. Evidence comes from different sources: Semilogarithmic plots of ($Na^+ + K^+$)-ATPase activity vs. the reciprocal temperature, usually referred to as Arrhenius plots, are biphasic, and can be approximated by two intersecting staight lines [6, 18,19].

Data from different kinds of experiments indicate a correlation between the phase transition temperature of the lipid matrix and the temperature at which the two linear portions of the logarithmic plot intersect. Among these methods are measurements of the fluorescence polarization of fluorescent probes attached to microsomes and right-angle light scattering from microsomal ATPase preparations and lipid extracts therefrom [20]. EPR measurements of spin-labeled membrane fractions and lipid extracts result in a similarly close correlation [21]. Also, from the pressure dependency of the enzymatic activity, similar conclusions can be drawn [22].

Another approach involves the use of different methods of more or less rigorous lipid depletion, possibly followed by subsequent experiments involving the restoration of lipid content. From these experiments it is obvious that for proper functioning of the ATPase lipids are needed. Though in many reactivation experiments the highest activities were obtained using phosphatidylserine, phosphatidylinositol, or phosphatidylglycerol, it is still unclear as to whether there really is a specific lipid requirement [23—28]. Moreover, there is evidence that only a limited number of lipid molecules in the direct vicinity of the enzyme are essential for its activity [29—31].

Materials and Methods

Chemicals

Alkali metal and alkaline-earth metal chlorides, ouabain and Tris were products of Merck, Darmstadt (NaCl and KCl 'Suprapur' grade, others 'Pro Analysi'). Enzymes, phospho*enol*pyruvate and ATP (disodium salt) were obtained from Boehringer, Mannheim. The enzymes, pyruvate kinase (EC 2.7.1.40, from rabbit muscle) and lactate dehydrogenase (EC 1.1.1.27, from pig muscle), were supplied in 50% glycerol, phospho*enol*pyruvate as the tricyclohexylammonium salt, and NADH as the disodium salt, grade I, 99%. The final sodium concentration brought about by the sodium salts of ATP and NADH amounts to about $3 \cdot 10^{-3}$ M, which is tolerable throughout all experiments.

Preparation of membrane fragments

The $(Na^+ + K^+)$-ATPase contained in membrane fragments was prepared from the grey matter of bovine brain. Bovine brain was obtained from the local slaughterhouse, and was worked up within a few hours. All manipulations were carried out in the cold (approx. 2—4°C). First, the starting material contained in 0.33 M sucrose (10^{-3} M EDTA) was crushed in a blender and subsequently centrifuged at 8000 rev./min in a Sorval GSA rotor to remove coarse contaminants. From the supernatant of this run, membranous material was spun down at 73 000 × *g* at 2°C. This high-speed centrifugation was repeated at least three times (90 min each) with intermediate homogenization in a Potter-Elvejhem homogenizer and resuspension of the pellets in sucrose solution. After the final run, the pellets were suspended in distilled water and were centrifuged again at 73 000 × *g* for 120 min. The pellets from this run were afterwards dispersed in water and subsequently freeze-dried. The lyophilysate was kept for times of up to 1 year without noticeable alterations at —20°C.

ATPase assay and enzymatic activity

To determine ATPase activity, the combined optical test using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes was employed. For the assay, a 'test solution' containing these enzymes and, in addition, phospho*enol*pyruvate and NADH in Tris-HCl buffer (pH 7.4) was mixed with the appropriate amounts of salt solutions and ATPase suspension. The reaction was started by the addition of ATP. At $\lambda = 366$ nm, the decrease in NADH, which is proportional to the ADP production, was followed. This decrease

was linear with time until all the phospho*enol*pyruvate had reacted.

$(Na^+ + K^+)$-ATPase activity is defined as the ADP production per unit time of the membrane suspension without ouabain minus the ADP production in the presence of 10^{-3} M ouabain. All activities are given in arbitrary units. The ATPase activities of different preparations range between 19 and 26 μmol ADP produced/mg total protein per h.

The ATPase suspension was made up from the lyophilized membrane preparation in 0.33 M sucrose (250 mg lyophilysate in 25 ml). The total volume was divided into aliquots, each of about 5 ml, which were kept frozen until used. During one set of experiments, suspensions from one stock were used. To correct for the temperature dependence of the buffer, equilibrium stock solutions of Tris-HCl buffer were prepared at 5, 10, 15, 20, 25, 30 and 35°C. All solutions were made up with Tris-HCl buffer (50 mM, pH 7.4).

Absorbance measurements and the temperature dependence of the ATPase reaction

The absorbance was measured with a Zeiss PMQ II spectrophotometer combined with a Zeiss TE-converter, and the extinction decay was recorded by an x-y recorder equipped with a scanner unit and a time base enabling the simultaneous monitoring of the absorbance and the temperature with respect to time. The temperature was measured by a thermocouple immersed in a second cuvette contained in a thermostatically controlled cuvette holder next to the test cuvette. The temperature was controlled and continually raised (0.5°C/min) by an automatic programmer connected to the thermostat. With this experimental set-up, the temperature can be both increased and recorded while the absorbance decays in proportion to the enzymatic reaction, proceeding at velocities which increase progressively as a result of the increasing temperature. The enzymatic activity at any recorded temperature can be read from the slope of the absorbance vs. time curve.

Results

The results presented here are based on measurements of the temperature dependence of $(Na^+ + K^+)$-ATPase activity under specified conditions of pH and electrolyte concentration.

As described under Materials and Methods, $(Na^+ + K^+)$-ATPase activity was measured over the temperature range from about 5 to about 35°C. In Fig. 1, some typical semilogarithmic plots of enzyme activity vs. reciprocal temperature ($1/T$) (Arrhenius plots) at various NaCl concentrations and at pH 7.4 are shown. In addition to the varying NaCl concentrations, KCl (16.7 mM) and $MgCl_2$ (1.95 mM) were present at constant concentrations throughout all runs. Each of these Arrhenius plots is biphasic, consisting of two linear portions. This is in agreement with previously reported findings [1,4,18—20]. The point of intersection of the two linear portions of the curve defines an 'inflection' temperature. This will be denoted as T_i throughout this paper.

As can be seen from Fig. 1, a change of the NaCl concentration displaces T_i, and also changes the slopes. From a number of such temperature runs at varied salt concentrations and constant pH, the salt dependence of the T_i

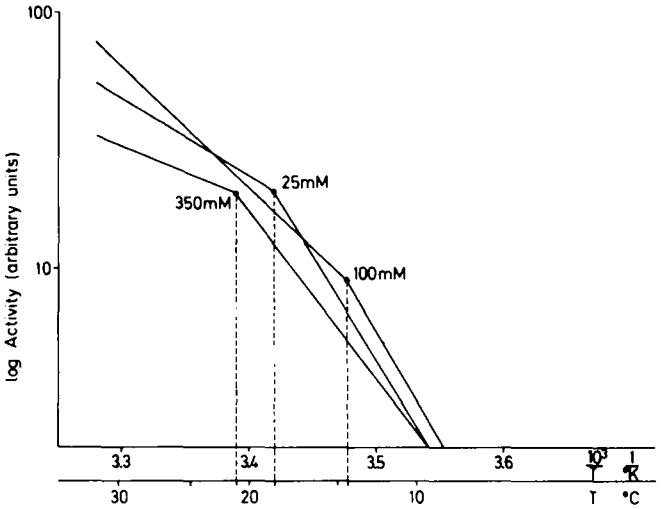

Fig. 1. Arrhenius plots of the enzymatic activity in the presence of 25, 100 and 350 mM NaCl. Conditions: pH 7.4 (Tris-HCl); 1.66 mM ATP; 1.95 mM $MgCl_2$; 16.7 mM KCl.

values and of the apparent energies of activation, E_a, at temperatures above and below T_i are obtained. In addition, such data contain information regarding the salt dependence of the enzymatic activity at any temperature between 5 and 35°C.

The quantity, $R \cdot \Delta \log A / \Delta(1/T)$, may be regarded as the apparent energy of activation (E_a) of the reaction, though this definition does not imply a coordination with the elementary steps of the reaction.

In Fig. 2a and b, T_i and E_a values at temperatures above and below T_i taken from such Arrhenius plots are plotted vs. NaCl concentration. Obviously, all of these quantities are markedly influenced by changes of the salt concentration. Interestingly, at the NaCl concentration (about 100 mM) where T_i passes through a minimum (Fig. 2a), both of the apparent energies of activation reach a maximum (Fig. 2b). E_a at $T > T_i$ is only about half that at $T < T_i$ throughout the whole range of salt concentrations measured.

In Fig. 2c, the enzymatic activity (in arbitrary units) is plotted vs. NaCl concentration. The activity values were taken from Arrhenius plots at different temperatures: one, well above T_i for all NaCl concentrations (29°C), and a second, below all T_i values at 10°C. Again at about 100 mM NaCl a maximum occurs. This means that in terms of enzymatic activity, NaCl at concentrations from 0 to about 100 mM activates the enzyme under the given conditions, whereas higher salt concentrations have an inhibitory effect on the enzyme.

In further experiments, the electrolyte concentration was changed by addition of divalent ions, i.e., Ba^{2+}, Sr^{2+}, Mg^{2+}, or Ca^{2+}. During these experiments, the NaCl concentration was kept constant at 100 mM, with KCl as before at 16.7 mM, pH 7.4. $MgCl_2$ was always present at a total concentration of at least 1.95 mM, and ATP at a total concentration of 1.66 mM was used.

T_i and the activation energies again depend on the salt concentration. The

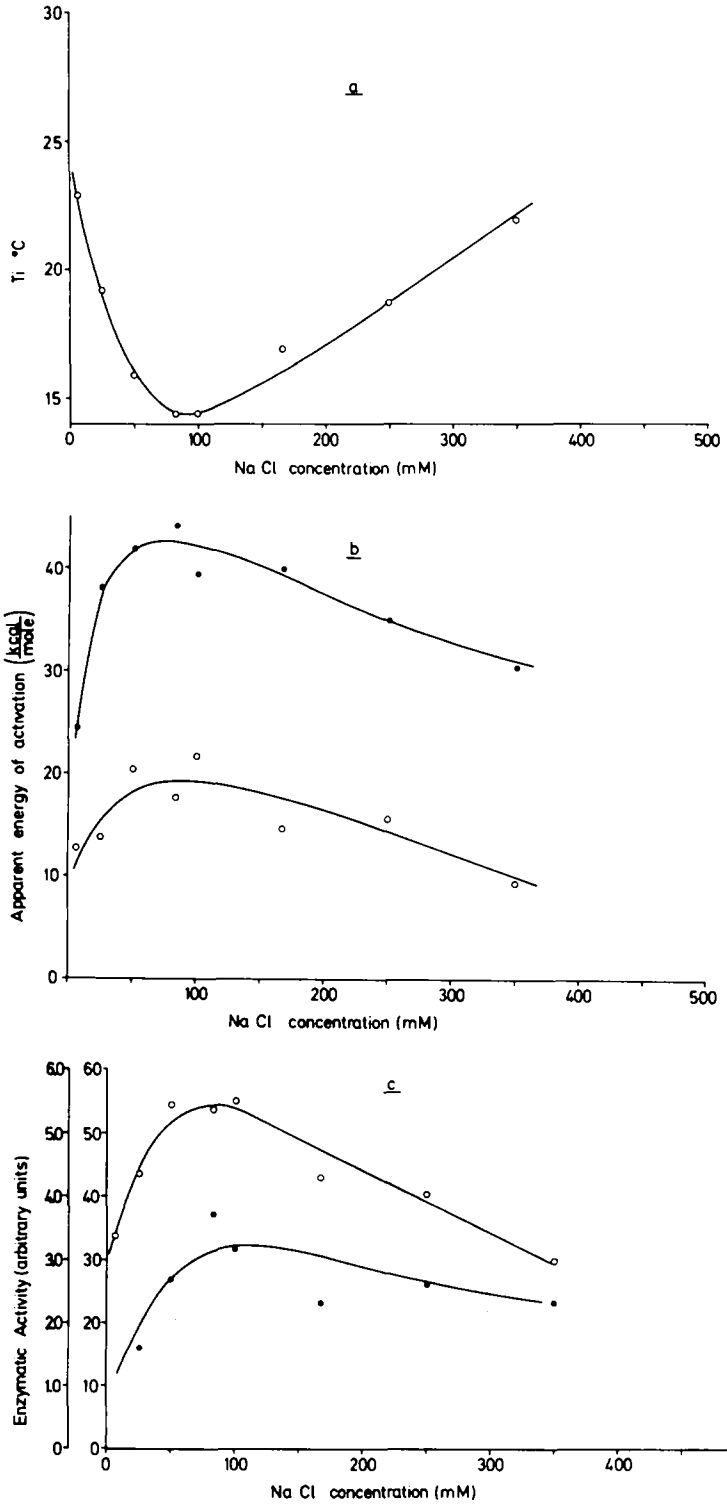

Fig. 2. NaCl dependence of: (a) T_i from the Arrhenius plots; (b) the apparent energies of activation: (●——●) at $T < T_i$, (○——○) at $T > T_i$; (c) ATPase activity: (○——○) at 29°C (inner ordinate), (●——●) at 10°C (outer ordinate). Conditions as in Fig. 1.

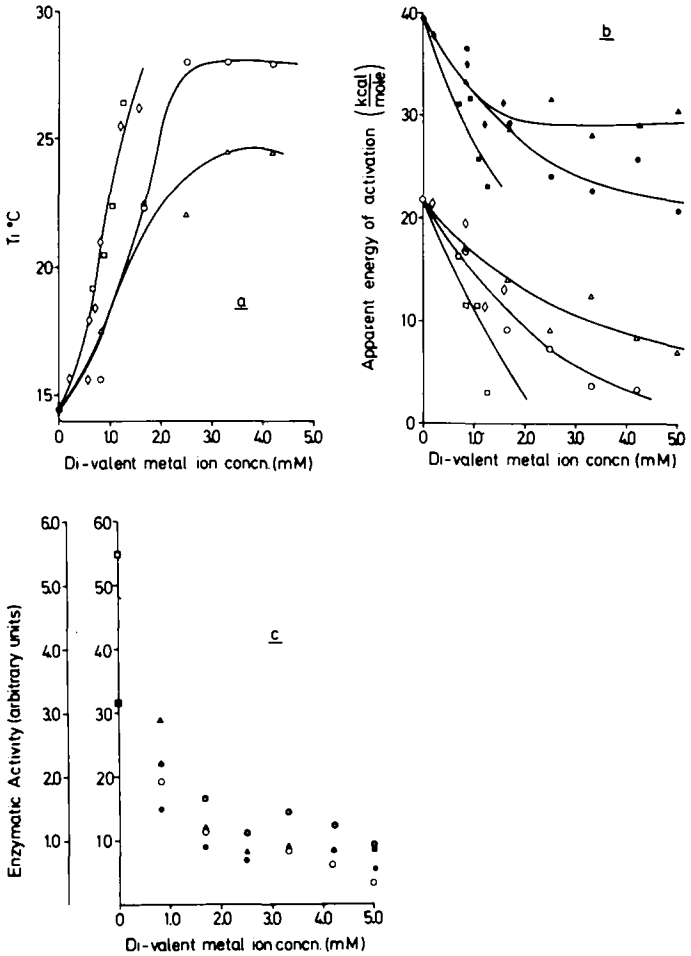

Fig. 3. Alkaline-earth metal ion dependence of: (a) T_i from the Arrhenius plots; (b) the apparent energies of activation: closed symbols $T < T_i$, open symbols $T > T_i$; (c) ATPase activity: open symbols 29°C (inner ordinate), closed symbols 10°C (outer ordinate). (○, ●) $SrCl_2$, (◇, ◆) $MgCl_2$, (△, ▲) $BaCl_2$, (□, ■) $CaCl_2$. Conditions: 100 mM NaCl, others as in Fig. 1.

results are given in Fig. 3a and b. However, as can be seen from these plots, the increase in T_i as well as the decrease in the activation energies occur within a much narrower concentration range than in the NaCl measurements.

The activities at 29 and 10°C (Fig. 3c) behave analogously to the other parameters. They decrease with increasing salt concentration, but this decrease is much more abrupt than with NaCl. This reflects the long-known inhibition of the ATPase by very small concentrations of divalent ions [32—36].

Discussion

The discussion of these results starts from the following points.

(1) The enzyme is considered to be situated within a charged surface formed by the negatively charged head groups of the surrounding lipids.

(2) The relationship between the surface potential Ψ_o, the surface charge density σ, and the bulk concentration c_i of solute ions with charge i is adequately described by the Gouy-Chapman theory of the diffuse double layer [37].

(3) As shown by theoretical considerations [11,16], which are based on the Gouy-Chapman theory, the phase transition temperature T_t^o of a charged membrane is lowered by ΔT_t to its actual value T_t as a result of increasing charge density σ at the surface. This effect may be counteracted by ionic screening at high bulk concentrations, c, of counterions.

$$T_t^o - \Delta T_t = T_t = T_t^o - \text{constant} \cdot \sigma \Delta f + \text{constant} \cdot \sqrt{c} \Delta f \qquad (1)$$

where Δf is the change of surface area during the phase transition.

Because of the close relationship between T_t of the lipids surrounding the enzyme and the temperature T_i, where the break in the Arrhenius plots occurs, it is supposed that an influence of the surface charge density similar to that on T_t may be observed for T_i. Information concerning this point may be obtained from inspection of Figs. 2a and 3a.

Since Mg^{2+} is known to bind to negatively charged lipid membranes, one would expect that under conditions of low 1 : 1 electrolyte concentration ($c_{NaCl} = 0$), Mg^{2+}, which was always present in the experiments, is complexed to the charged lipids. Thereby, the surface charges become neutralized and the highest attainable value of T_i should result according to Eqn. 1.

Addition of NaCl will reduce the degree of Mg^{2+} binding, and Mg^{2+} will be released from the surface. This will cause a successive increase in the charge density σ resulting in a decrease in T_i (cf. Eqn. 1). This process should continue till no further Mg^{2+} can be displaced from the surface by addition of NaCl. Then σ will become constant, and correspondingly, T_i will no longer decrease. This behaviour is exactly that shown by the curve in Fig. 2a at $0 < c_{NaCl} \lesssim 80$ mM. From this concentration on, where σ becomes constant, the shift of the phase transition temperature is solely determined by ionic screening. This predicts that T_i will again increase when the electrolyte concentration is raised further. As is obvious from Fig. 2a, at higher electrolyte concentrations T_i indeed adopts higher values again, finally approaching its initial value.

This analysis of the electrolyte dependence of T_i reveals at least a qualitative agreement between the behaviour of T_i and that deduced for the phase transition temperature of a charged membrane in the general case.

The results in Fig. 3a will be discussed in a later section, since the effects of divalent ions demand some detailed comments. However, it may be said in advance that the effect exerted on T_i by divalent ions is equivalent to that found with NaCl. Equally with KCl instead of NaCl, essentially equivalent results have been obtained. Investigations of other alkali metal ion dependencies are forthcoming.

The increase in T_i at NaCl concentrations greater than about 100 mM, however, is considerably steeper than has been reported for vesicles obtained from pure methylphosphatidic acid [11]. This finding can be explained by two different lines of argument, and at present, a decision in favour of either is not possible.

If at higher temperatures the affinity between protein and lipids is assumed

to decrease, an increase in the temperature would reduce the number of lipid molecules attached to the protein. For this reason, with increasing temperature, the total change of lipid density sensed by the enzyme is much greater than if only the expansion of the area occupied by the lipid molecules had been accounted for. In this case, the steep increase in T_i at high salt concentrations would be brought about by a much higher value of Δf compared to the model case (cf. Eqn. 1).

Thus, the quantitative difference between the experimental results from the model and the real system have been attributed to an appropriate definition of the 'effective' area occupied by the lipid molecules. This point of view, however, implies the applicability of the Gouy-Chapman theory, which appears fairly questionable in this case, where the surface charges would become separated by a considerable distance.

As an alternative explanation, 'intrafacial' hydrogen bonding of the charged lipid head groups to donors in the membrane surface might be considered. This has been discussed in a recent publication [38]. It has been shown that under suitable conditions, intrafacial hydrogen bonds can make a significant contribution to the free energy of the membrane. This causes an elevation of the transition temperature. In the present case, it is conceivable that the hydration of the surface becomes increasingly disturbed with increasing electrolyte concentration, and accordingly, the electrical double layer will become more condensed. This would result in an increasing tendency to form hydrogen bonds within the surface. This in turn would cause a stabilization of the membrane structure, which would become manifest by a gradual increase in the transition temperature as the electrolyte concentration is raised.

An even more direct influence of electrostatic conditions at the membrane surface is perceptible in the apparent energies of activation, E_a (Fig. 2b). Since the substrate of the enzymatic reaction is an anionic species, either $MgATP^{2-}$ or ATP^{4-}, its approach to the negatively charged surface is hindered by an energy barrier due to the surface potential. The enzyme, on the other hand, is fixed within the membrane. Therefore, the probability of encounters between the substrate and the enzyme is reduced owing to the repulsive effect of the surface charge towards the substrate. Hence, an activation of substrate molecules by an amount of energy equal to at least that which is necessary to counteract the surface potential is an absolute requirement for the reaction to take place. Therefore, an extra amount of energy has to be afforded as a prerequisite for the reaction in addition to the intrinsic energy of activation. The latter quantity includes those contributions to the total energy of activation which result from the elementary steps of the chemical reaction between the enzyme and its substrate. Therefore, the reaction is not affected, or at most, only indirectly affected by the ionic conditions in the solution, whereas the additional contribution is by definition of purely electrostatic origin, and changes proportionally to the surface potential.

As pointed out in the foregoing section, while NaCl is raised from 0 to about 80 mM, Mg^{2+} is released from the surface, which consequently becomes successively more negative. A further increase in the NaCl concentration has been seen to cause gradually increasing screening. An increase in the charge density at the surface, however, is equivalent to an increased surface potential, and

increased screening on the other hand is identical with a reduced surface potential. Therefore, a corresponding increase or decrease, respectively, of the total energy of activation must be the consequence.

An estimate may give further support to the above arguments: It is a reasonable assumption that the surface potential increases by $\Delta\Psi_o = 150$ mV when the NaCl concentration is raised from 0 to approx. 100 mM. For 1 mol this would correspond to a change of $\Delta E_a = N_L z \cdot e\Delta\Psi_o$ of the potential energy in the case of z-fold charged ions. (N_L, Avogadro's number; e, the elementary charge). With $z = 4$, $\Delta E_a = 14$ kcal/mol is calculated. In the experimental curves (Fig. 2b), $\Delta E_a \simeq 19$ kcal/mol at $T < T_i$ and $\Delta E_a \simeq 12$ kcal/mol at $T > T_i$. This is in good agreement with the above estimate. The difference between the two values of ΔE_a at $T < T_i$ and $T > T_i$ may be attributed to different surface charge densities in both states. These results might suggest that ATP^{4-} ($z = 4$) is the substrate rather than $MgATP^{2-}$, although in the bulk solution most of the ATP will be present in the complexed form. What remains unexplained by these considerations is the sudden change in the activation energy at T_i. This effect, which is identical with the occurrence of biphasic Arrhenius plots, is not caused by the electrostatic contribution to the energy of activation. Rather, it results from other factors, such as the motional freedom within the hydrophobic part of the membrane, which may influence the intrinsic energy of activation.

The activity is enhanced in parallel with the activation energies when NaCl is raised from 0 to approx. 100 mM and it is reduced by further increases in salt concentration (see Fig. 2c). Thus, the relatively highest activities are obtained where the energy of activation is also high. At first sight this is somewhat surprising. The only likely reason for this behaviour is an influence of the surface electrostatics upon the pre-exponential factor of the Arrhenius equation. In addition, it is also inferred from the data that this influence is sufficiently large to overcome the inhibiting effect caused by the elevated energy of activation. The reaction under consideration deals with ionic species, and since the pre-exponential factor is related to the entropy of activation, an influence of surface electrostatics is quite plausible. Nothing is known about the configuration of the transition state, but whatever it might be, it is quite obvious that charges are involved, and therefore that it will be sensitive to changes of the ionic environment.

Similar experiments have been undertaken in the presence of alkaline-earth metal ions instead of Na^+ (Fig. 3a—c). Results from these measurements should help in distinguishing between the mechanism proposed here, and the possibly more common view that the interaction of ions relevant for the reaction takes place at the protein.

As pointed out by McLaughlin et al. [9], the screening ability of divalent metal ions is orders of magnitude higher than that of monovalent ions. As a consequence of the Gouy-Chapman theory, either monovalent ions at concentration c_+ or divalent ions at c_{++} give rise to an identical screening effect, if:

$$c_+ = \text{constant} \cdot \sigma\sqrt{c_{++}} \qquad (2)$$

Thus, assuming $\sigma = 1$ negative charge/38 Å^2, the same screening may be attained with monovalent ions at a concentration of $c_+ = 100$ mM or divalent

ions at $c_{++} = 0.2$ mM. Provided that the electrostatic concept is indeed adequate for the ATPase system, these considerations should also be applicable. Then it is expected that in the presence of Ba^{2+}, Sr^{2+}, Mg^{2+}, or Ca^{2+}, the same effects that have been observed with Na^+ will occur already at considerably lower concentrations. At least qualitatively the experimental results given in Fig. 3a—c corroborate this prediction. To enable comparison of results from experiments in the presence of alkaline-earth metal ions with those made in the presence of Na^+, the concentrations c_{++} of divalent ions actually present in the solution have been converted into 'electrostatically equivalent' concentrations c_+ of monovalent ions. The calculations were performed assuming the following premises. (1) Mg^{2+} forms complexes wtih ATP^{4-} with a stability constant of $K = 10^4\ M^{-1}$ [39]. (2) The surface charge density is taken as constant at σ = 1 negative charge/40 Å^2, and complex formation between divalent ions and lipids is not taken into account. According to the first premise, nearly all of the ATP (1.66 mM) present is bound in the complex $MgATP^{2-}$, thus reducing the concentration of Mg^{2+} to an effective concentration of free Mg^{2+} (c_{Mgfree}). Throughout all experiments, where the total concentration is $c^o_{Mg} = 1.95$ mM, the concentration of free Mg^{2+} is $c_{Mgfree} = 0.55$ mM. For the calculation of a concentration c_+ that is electrostatically equivalent to c_{++}, one has to take into account that the experiments carried out at varying NaCl concentrations were made in the presence of $c_{Mgfree} = 0.55$ mM, and that those at varying concentrations of divalent ions were performed in the presence of 100 mM NaCl. For this reason, Eqn. 2, which is confined to conditions under which ions of only one valency type are present at a time, is not directly applicable. Instead, the calculation starts from the more comprehensive Graham equation (see, for example, Ref. 9). From this equation, firstly the potentials at increasing c_+ but at constant c_{++} (0.55 mM) have been evaluated. Since these calculations have been made under the simplifying assumption that only 1 : 1 and 2 : 2 electrolytes but no mixed (2 : 1) electrolytes were present, the result does not exactly meet the real conditions. However, it can be estimated that this causes only minor deviations which may be neglected. In the next step, the potentials at constant $c_+ = 100$ mM and varying concentrations $c_{++} = 0.55 \cdot 10^{-3}\ M + c^o_{Ba,Sr}$ were calculated, where $c^o_{Ba,Sr}$ is the total concentration (M) of either Ba^{2+} or Sr^{2+}.

The complexation between Ba^{2+} or Sr^{2+} and ATP^{4-} has been ignored. This is justified for two reasons. (1) The stability constant for Ba^{2+}- and Sr^{2+}-ATP complexes is lower by nearly one order of magnitude than that of Mg^{2+}- and Ca^{2+}-ATP [39]; (2) it is only the sum fo free Mg^{2+} and Sr^{2+} or Ba^{2+} that enters into the calculation.

A comparison of the potentials calculated from both procedures finally yields c_+ values corresponding to each value of $c_{++} = 0.55 \cdot 10^{-3}\ M + c^o_{Ba,Sr}$. The results are used in Fig. 4a—c. Here the data obtained with $BaCl_2$ and $SrCl_2$ (Fig. 3a—c) are replotted over the calculated c_+ values.

With this method, the T_i curves, the activation energy curves and the activity curves become expanded to nearly the same width as that measured at variable NaCl concentrations.

From a practical point of view, the most noteworthy effect of the transcription from c_{++} to c_+ is that on the enzymatic activity. Divalent ions are

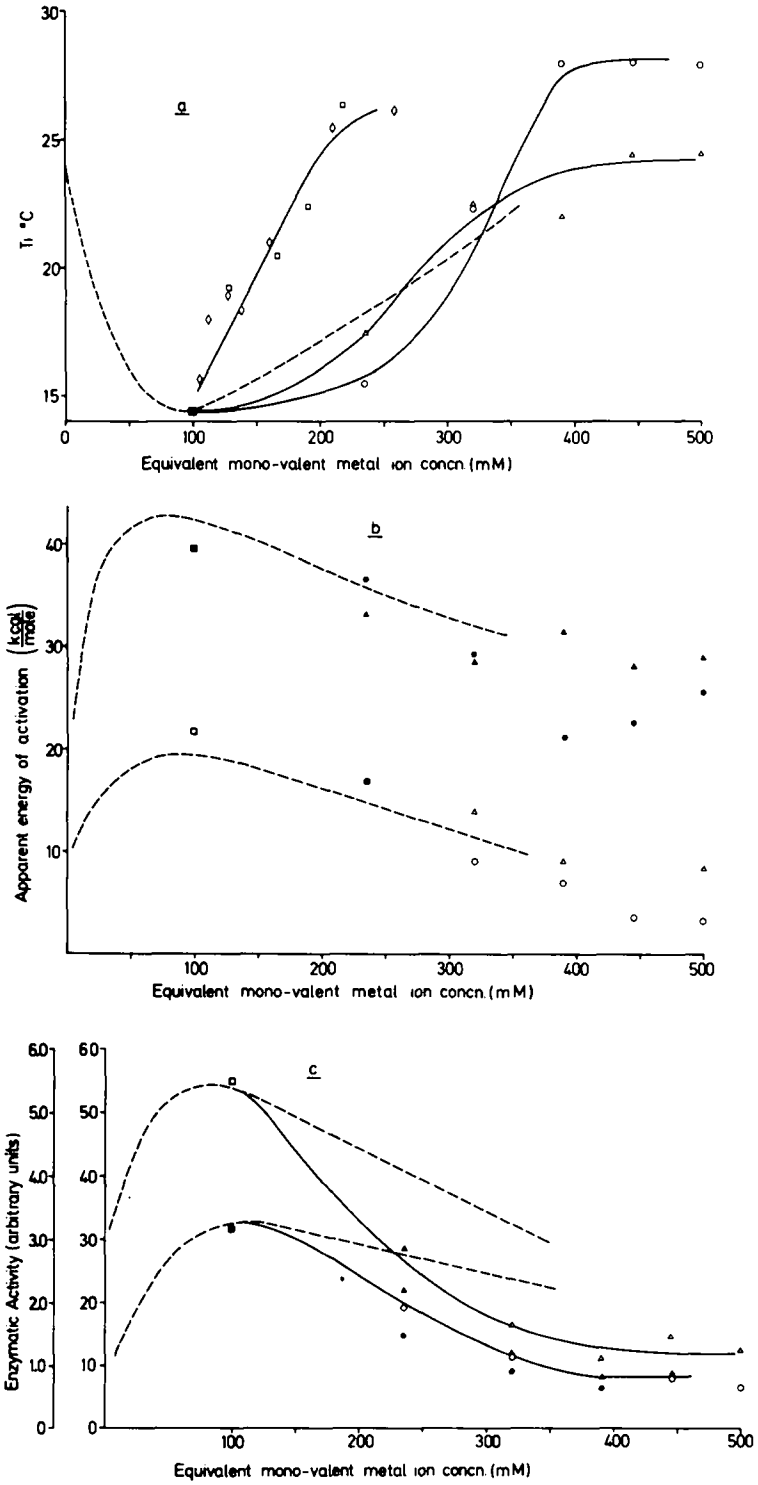

Fig. 4. Dependence upon the calculated 'equivalent' monovalent metal ion concentration of: (a) T_i from the Arrhenius plots; (b) the apparent energies of activation: $T < T_i$ closed symbols, $T > T_i$ open symbols; (c) the enzymatic activity: open symbols, 29°C (inner ordinate); closed symbols, 10°C (outer ordinate). Dashed lines: see Fig. 1. Symbols as in Fig. 3.

usually claimed to be potent inhibitors of the ATPase, and this fact is ascribed to a competition for Mg^{2+}-binding sites which are postulated to be situated on the protein. However, expanding the concentration scale as was done here 'raises' the activity to, or nearly to, its 'normal' values found at moderately low salt concentrations.

At 'equivalent' concentrations, $c_+ \gtrsim 380$ mM, T_i, the activation energies and the activities adopt constant values so that the curves level off to a plateau. It is not clear why $BaCl_2$ gives rise to higher T_i values than $SrCl_2$. This effect, however, appears also in the energies of activation and the activities, and hence it must be considered to be of ion-specific origin. One might speculate that slight differences of the structure of the screening layer, perhaps because of differences in the hydration of ions, are responsible. The remaining slight deviations of the $BaCl_2$ and $SrCl_2$ curves from the NaCl curve cannot be fully explained.

Nevertheless, the seemingly immense dissimilarity between the effectivities of mono- and divalent ions has successfully been abolished by replacing the concentration of ions by their screening ability. These results yield strong evidence in favour of the 'electrostatic concept' of the ATPase reaction. When Mg^{2+} and Ca^{2+} are used, complexation between the metal ions and ATP has to be taken into account. The free ion concentrations have been calculated using $K = 10^4\ M^{-1}$ for the stability constant of the Mg^{2+}- as well as for the Ca^{2+}-ATP complex. In all other respects, the conversion of c_{++} into corresponding c_+ values was carried out as before.

In principle, Mg^{2+} and Ca^{2+} behave similarly to Ba^{2+} and Sr^{2+} (cf. Fig. 4a—c). However, the conversion of c_{++} into corresponding c_+ values in these cases does not expand any of the curves to the full width of the NaCl curves. This may be attributed to complex formation between the metal ions and the surface lipids, which had been disregarded up to this point. Increasing binding of Ca^{2+} or Mg^{2+} will cause the surface charge density to become successively smaller. This renders the assumption of $\sigma = 1$ charge/40 Å^2 implicit in the calculations incorrect in this case. Inserting lower surface charge densities into the calculations tends to expand the curves further.

The finding that Ca^{2+} and Mg^{2+} on the one hand and Sr^{2+} and Ba^{2+} on the other behave similarly and that Ca^{2+} and Mg^{2+} obviously form complexes with the membrane surface is in accordance with the results reported by McLaughlin et al. [9]. The results of Kimelberg [40] on phosphatidylserine-reactivated ATPase in the presence of Mg^{2+}, in excess of ATP, are similar to the results of this work. He also suggests that acidic phospholipids are responsible for the effects of Mg^{2+} upon the ATPase. The interpretation, however, is based exclusively on the assumption that Mg^{2+} acts via fluidity changes. Hence, the explanation of the fact that at high Mg^{2+} concentration (10 mM) the slope of the Arrhenius plot becomes less steep, though at the same time T_i is shifted to high values, is rendered problematic.

Concluding Remarks

The fundamental postulate of this work is that electrostatic membrane properties are capable of controlling the behaviour of membrane enzymes.

This has been illustrated for the $(Na^+ + K^+)$-ATPase, though the detailed molecular mechanism has not been dealt with.

Beyond the consistency with experimental findings some more cognitive arguments are also in favour of the hypothesis. (1) An influence of the surrounding lipids on the enzyme, irrespective of the mechanism, can be considered as a matter of fact. Since polar lipids are known to be subject to ionic influences, a feedback to the enzyme is strongly suggested. Moreover, it would be rather difficult to imagine its absence. (2) The current explanation of the influence of ions upon the enzyme only in terms of an interaction between the ions and the protein requires the postulate of numerous different highly specific binding sites for each kind of ion [32,34]. For instance, separate binding sites for Na^+ and K^+, or even more than one for each ionic species, have been postulated.

In contrast to this highly involved and complicated view, a much more unconstrained view of the interaction of the ATPase with ions is obtained if the surrounding lipids are included in the considerations. Thus, a more general picture of the function of the $(Na^+ + K^+)$-ATPase develops. Moreover, it might be suggested that this view could be of still greater universality, in as much as the ion specificities of different kinds of ATP-hydrolyzing enzyme (cf. Ca^{2+}- and Mg^{2+}-ATPases) might possibly be assigned rather to the lipids than to differential characteristics of the proteins.

Since all the data in this work have been acquired with particulate material, conclusions may be drawn only with respect to the enzyme, and no direct information concerning the transport function of the $(Na^+ + K^+)$-ATPase can be obtained. However, an extrapolation of the conclusions of this work to the ATPase acting in an undisrupted cell membrane might be adequate.

Most cell membranes have been found to be built up by an assymmetrical lipid bilayer, where both layers differ in their lipid composition [41]. This also implies an asymmetrical charge distribution. It has been shown [42] that when there exists an asymmetry of charges, which is equivalent to an asymmetry of surface potentials across the membrane, alterations of the local charge distribution in one of the bilayers will induce a corresponding change in the other layer. Therefore, it is conceivable that ion transport across the membrane might be triggered by the same mechanism, which acts on the enzyme in the particulate preparation.

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