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THE EFFECT OF pH, OF ATP AND OF MODIFICATION WITH PYRIDOXAL 5-PHOSPHATE ON THE CONFORMATIONAL TRANSITION BETWEEN THE Na^+-FORM AND THE K^+-FORM OF THE ($Na^+ + K^+$)-ATPase

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An increase in pH decreases the Na^+ concentration ($Na^+ + K^+ = 150$ mM) necessary for half-maximum activation of the ($Na^+ + K^+$)-ATPase at non-saturating concentrations of ATP just as an increase in the concentration of ATP at a given pH. It also decreases the concentration of Na^+ necessary for transformation from the K^+-form to the Na^+-form at equilibrium conditions ($Na^+ + K^+ = 150$ mM). An increase in pH increases the rate of the transformation from the K^+-form to the Na^+-form of the system and decreases the rate of the reverse reaction.

The pH effect on the conformation suggests that the K^+-form is a protonated form and the Na^+-form a deprotonated one. The similarity between the effect of an increase in pH with non-saturating concentrations of ATP and that of an increase in ATP at a given pH suggests that ATP exerts its effect on the transformation from the K^+- to the Na^+-form by a decrease in pK values of the system, i.e., by releasing protons, a Bohr effect. Enzyme modified by reaction with pyridoxal 5-phosphate terminated by $NaBH_4$ behaves at a given pH as if it were non-modified enzyme but at a higher pH. The 'pH effect' is seen after modification by pyridoxal 5-phosphate in the presence of ATP, of Na^+ without and with ATP, of K^+ with ATP but not in the presence of K^+ alone. The modification has also a 'pH effect' on the rate of the transformation from the K^+-form to the Na^+-form and on the reverse reaction. There are at least two different pyridoxal 5-phosphate-reactive groups (amino groups), one which can be protected by ATP and which is of importance for activity and another which is not protected by ATP and which is of importance for the pH effect on the conformation. The effect of a protonation-deprotonation of amino groups on the conformation is explained by an involvement of the amino groups in salt bridge formation inbetween and inside the polypeptide chains, a hemoglobin-like situation. The protonated K^+-form is then a tense T-structure with a high K^+, low Na^+ affinity and the deprotonated Na^+-form a relaxed, R-structure with high Na^+, low K^+ affinity. ATP facilitates deprotonation by decreasing pK values. Oligomycin has 'pH effect' on the $K_{0.5}$ for Na^+ under equilibrium and steady-state conditions, but oligomycin has no effect on the rate of the transformation from the K^+-form to the Na^+-form, but gives a pronounced decrease of the rate of the reverse reaction, indicating that oligomycin does not react with the K^+-form but with the Na^+-form of the system and prevents the protonation, the E_1 to E_2 transformation.

Introduction

It is characteristic for the ($Na^+ + K^+$)-ATPase that it can discriminate between K^+ and Na^+ [1] and that in the presence of K^+ it exists in a conformation (K^+-form) which is different from the conformation in the presence of Na^+ (Na^+-form) [2–8].

In other words, the system can sense the difference between K^+ and Na^+ and the molecular

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structure has the ability to adapt to the difference. This is apparently the basis for the ability to discriminate.

The adaptation of the molecular structure to K^+ and Na^+, respectively, is intimately connected to a change in the affinity for ATP [2,3,5,9]. When the structure is adapted to Na^+, a high-affinity site for ATP is opened, while when adapted to K^+ the affinity for ATP is decreased (or the site is closed?). This means that ATP will tend to change the structure from the K^+-form to the Na^+-form. It is observed as an increase in the rate of the transformation of the K^+-form to the Na^+-form by ATP at a given Na^+/K^+ ratio [5,6]. And under steady-state conditions as a decrease in the Na^+ concentration for half-maximum Na^+ activation in the presence of K^+ ($Na^+ + K^+ = 150$ mM) when the ATP concentration is increased [10].

But what is the mechanism behind the adaptation of the molecular structure to the cations?

An increase in pH shifts the equilibrium between the K^+-form and the Na^+-form towards the Na^+-form at a given Na^+/K^+ ratio [8], and under steady-state conditions decreases the Na^+ concentration for half-maximum Na^+ activation of hydrolysis with non saturating concentrations of ATP [11], just as an increase in ATP at a given pH [10]. This indicates that a deprotonation-protonation reaction is involved in the transformation between the two structures and that the K^+-form is a protonated while the Na^+-form is a deprotonated structure. This suggests that the ATP effect on the transformation is due to a decrease in pK, and that ATP thereby facilitates the deprotonation which leads to a transformation from the K^+-form to the Na^+-form, a Bohr effect.

In the present paper the pH effect has been further investigated and compared to the ATP effect. By modification of the system with pyridoxal 5-phosphate an attempt has been made to locate groups on the system involved in the deprotonation-protonation reaction.

Methods

Enzyme was prepared from the rectal glands of *Squalus acanthias* and tested as previously described [12]. The specific ATPase activity was 1462 ± 33 (1267–1632) ($n = 12$) μmol P_i/mg protein per h and the specific *p*-nitrophenylphosphatase activity 213 ± 4 (182–234) ($n = 12$) μmol nitrophenol/mg protein per h.

With low ATP concentrations the enzyme activity was tested by measuring the amount of ^{32}P released from ATP labelled with ^{32}P in the γ-position according the methods by Lindberg and Ernster [13]. ^{32}P was counted in a liquid scintillation counter. With ATP concentrations of 500 μM or higher the activity was tested by measuring the P_i released by the method of Fiske and SubbaRow [14].

[^{32}P]ATP was from Amersham International, Amersham, U.K. ATP was from Böhringer. [^{32}P]ATP and ATP were purified and converted to the Tris salt by chromatography on a DEAE-Sephadex A-25 (Pharmacia) column [2].

Eosin was used as the fluorescence probe for testing the changes in conformation [9].

The rate of the transfer of the enzyme from the K^+-form to the Na^+-form and vice versa was measured in a stop-flow apparatus with a mixer constructed according to Berger et al. [15]. It was connected to a Perkin-Elmer MPF 44 A spectrofluorometer with the electronics slightly modified in order to give a fast enough reaction. Each of the two syringes in the stop-flow apparatus contained 3.4 ml. The injection time was 76 ms. The flow velocity was 17 m/s and the distance from mixer to mid-point of the 'cuvette' was 12 mm and the diameter 2.6 mm. The reaction was monitored by the change in fluorescence of eosin; excitation at 530 nm, emission at 560 nm, slitwidth 10 nm both for excitation and emission.

In the reaction with pyridoxal 5-phosphate the enzyme was preincubated with the ligands for the time shown on the figures at 22°C in a 30 mM *N*-ethylmorpholine-HCl buffer at pH 7.4 in the dark. The incubation was terminated by addition of an excess of $NaBH_4$ and the enzyme was washed three times by centrifugation and resuspended in a 30 mM histidine-HCl buffer, pH 6.8 with 25% glycerol and stored at −20°C until used.

Protein was determined by the method of Lowry et al. [16] with albumin as standard.

Results

When pH is increased from 7.4 to 8.4 the distribution between the K^+-form and the Na^+-

form of the $(Na^+ + K^+)$-ATPase at a given Na^+/K^+ ratio ($Na^+ + K^+ = 150$ mM) is shifted towards the Na^+-form.

This is observed under equilibrium conditions at 22°C with no ATP when the distribution between the K^+-form and the Na^+-form of the molecules is measured as a function of the Na^+/K^+ ratio (Fig. 1A). The fluorescence of eosin is used to monitor the change in configuration.

The effect of an increase in pH on the Na^+ vs. K^+ titration curve is also observed under steady-state conditions with a non-saturating concentration of ATP (0.1 μM) when the hydrolysis of ATP is used to monitor the effect (Fig. 1B). An increase in pH decreases the concentration of Na^+ necessary for half-maximum Na^+ activation ($K_{0.5}$ for Na^+) in the presence of K^+ ($Na^+ + K^+ = 150$ mM). This is similar to the effect of an increase in ATP at a given pH (Fig. 1C, compare Fig. 1B). The decrease in $K_{0.5}$ for Na^+ activation of hydrolysis is due to an effect of ATP as such and not to the hydrolysis of ATP [10]. The steady-state effect of the pH increase from 7.4 to 8.4 on the $K_{0.5}$ for Na^+ is comparable to an increase in the ATP concentration to 4 μM at pH 7.4 (compare Fig. 1B and Fig. 7A).

ATP increases the rate of the transfer of the K^+-form into the Na^+-form [5,6] and, as seen from Table I and Fig. 2, an increase in pH also increases the rate of this transfer. Tested under identical ionic conditions, the time for half-maximum response, $t_{1/2}$, for the transfer from the K^+-form to the Na^+-form is decreased 5–6-times by an increase in pH from 7.4 to 8.4 at 22°C (Fig. 2 and Table I), and at 4°C (Table I).

There is also an effect on the reverse reaction, the transformation from the Na^+-form to the K^+-form. The $t_{1/2}$ is increased 4-times by an increase in pH from 7.4 to 8.4 at 22°C and 1.8-times at 4°C. The effect of temperature on this rate is thus less pronounced at pH 8.4 than at 7.4.

The $t_{1/2}$ for the transformation from the K^+-form to the Na^+-form (from 1 mM K^+ to 0.5 mM K^+ final, 75 mM Na^+) at pH 7.0 was about 2.7 s at 23°C in experiments where the intrinsic fluorescence of tryptophan has been used to monitor the conformational changes [6]. 10 μM ATP increased the rate 4–5-times (read from Fig. 5 in Ref. 6). This is to be compared with the 5–6-times increase

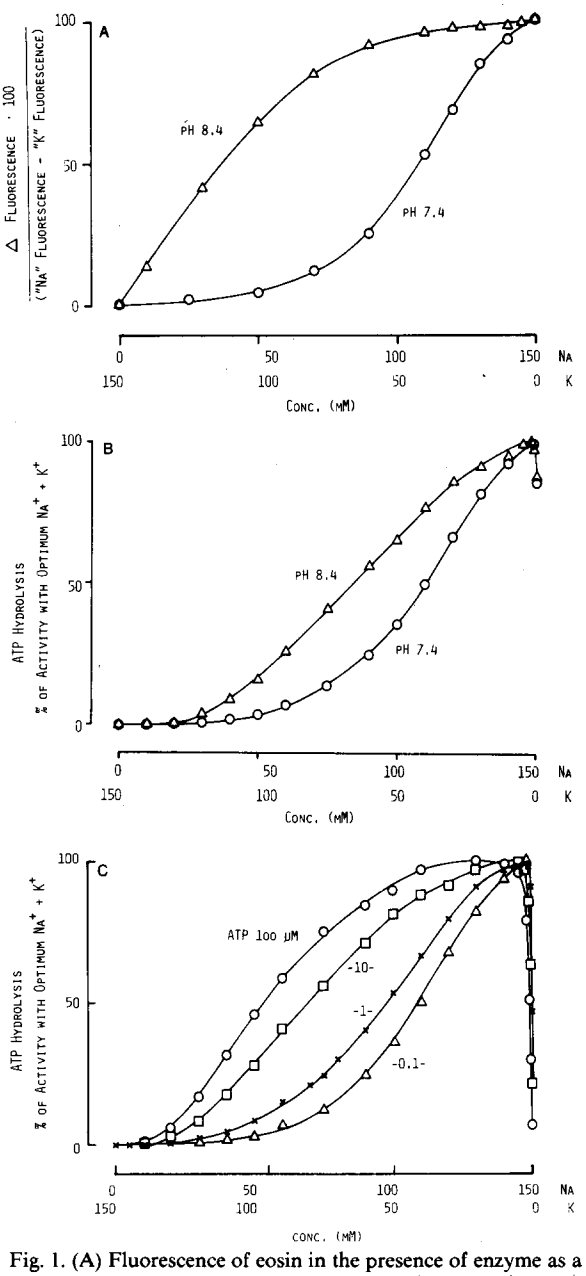


Fig. 1. (A) Fluorescence of eosin in the presence of enzyme as a function of different combinations of Na^+ and K^+ ($Na^+ + K^+ = 150$ mM) at pH 7.4 and 8.4, respectively, 22°C. 100 μg $(Na^+ + K^+)$-ATPase/ml, 0.1 μM eosin Y. 30 mM histidine-HCl at pH 7.4, 30 mM Tris-HCl at pH 8.4 as buffer. (B) Activation by $Na^+ + K^+$ ($Na^+ + K^+ = 150$ mM) of ATP hydrolysis at pH 7.4 and 8.4. 0.1 μM ATP, 50 μM Mg^{2+}, buffers as in (A). (C) Activation by $Na^+ + K^+$ ($Na^+ + K^+ = 150$ mM) of the hydrolysis of ATP with 0.1, 1, 10, and 100 μM ATP, respectively; the Mg^{2+} concentration were 50, 100, 100 and 500 μM, respectively, 30 mM histidine-HCl buffer, pH 7.4, 37°C. The activity is give as percent of the maximum obtainable with the given ATP concentration.

TABLE I

THE HALF-TIME, $t_{1/2}$, IN SECOND FOR TRANSFORMATION FROM THE K^+-FORM TO THE Na^+-FORM OF THE ENZYME (AND VICE VERSA) AT pH 7.4 AND 8.4 AT 22°C AND AT 4°C

For experimental conditions for the transformation from the K^+-form to the Na^+-form see Fig. 2. For the transformation from the Na^+-form to the K^+-form, syringe 1 contained 30 mM histidine-HCl at pH 7.4 and 30 mM Tris-HCl at pH 8.4, and at each pH 20 mM Na^+, 0.5 μM eosin Y, 90 μg enzyme. Syringe 2: the same buffer as in syringe 1, 20 mM Na^+, 40 mM K^+, 0.5 μM eosin Y. Time delay zero at 22°C, 7 ms at 4°C. With +oligomycin the solution in both syringes contained 10 μg oligomycin per ml, added in 5 μl alcohol. Each value is based on at least three different experiments and for each experiment on three to five curves. The 0.019-s value for $t_{1/2}$ for the transformation from the Na^+-form to the K^+-form at pH 7.4, 22°C is of the order of the $t_{1/2}$ for the release of the eosin probe from the Na^+-form and may therefore be a maximum value for the rate of transformation.

Temp. (°C)	pH	$t_{1/2}$			
		K^+-form → Na^+-form		Na^+-form → K^+-form	
			+oligomycin		+oligomycin
22	7.4	0.53 ±0.01	0.53 ±0.01	(0.019±0.001)	1.92±0.02
	8.4	0.100±0.002	0.093±0.003	0.078±0.003	2.08±0.14
4	7.4	4.18 ±0.07	3.75 ±0.12	0.101±0.001	15.5
	8.4	0.69 ±0.03	0.65 ±0.02	0.183±0.006	28.8 ±0.25

in rate by an increase in pH from 7.4 to 8.4 and which has an effect on $K_{0.5}$ for Na^+ under steady-state conditions comparable to the effect of an increase in the ATP concentration to 4 μM at pH 7.4.

There are no measurements on the effect of ATP on the rate of the transformation from the Na^+-form to the K^+-form, so it is unknown whether ATP decreases this rate of transformation as is observed by an increase in pH.

An increase in pH has thus an 'ATP effect' not only on the $K_{0.5}$ for Na^+ activation, but also on the rate of the transformation from the K^+-form to the Na^+-form supporting the view that the ATP effect on the transformation is related to a deprotonation of the system, a decrease in pK values.

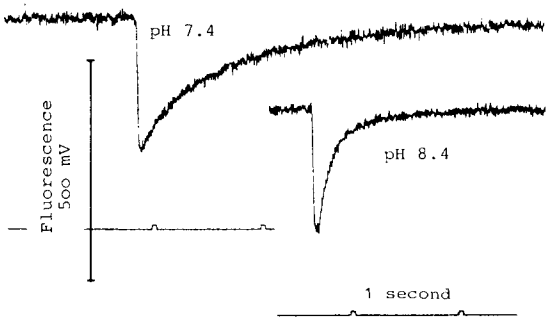


Fig. 2. The effect of pH on the rate of transformation from the K^+-form (low fluorescence) to the Na^+-form (high fluorescence). Syringe 1 of the stop-flow apparatus contained at pH 7.4, 30 mM histidine-HCl and at pH 8.4, 30 mM Tris-HCl, and at each pH 2 mM K^+, 90 μg ($Na^+ + K^+$)-ATPase/ml, 0.5 μM eosin Y. Syringe 2: buffers as in syringe 1, 2 mM K^+, 80 mM Na^+, 0.5 μM eosin Y. Each syringe contained 3.4 ml. Injection time, 76 ms. The time delay was set at 7 ms. Slit width 10 nm, excitation at 530 nm, emission 560 nm.

Pyridoxal 5-phosphate

There are two H^+ dissociation constants involved in the transformation from the K^+-form to the Na^+-form, one with a pK between 5 and 7, which may be histidine groups and another with a pK higher than 9 which may be ϵ-amino groups on lysine [8]. As pyridoxal 5-phosphate reacts with ϵ-amino groups on lysine [17], how a reaction of the enzyme with pyridoxal 5-phosphate influences the equilibrium between the K^+-form and the Na^+-form and the rate of the transformation from the one to the other has been studied in order to see if pyridoxal 5-phosphate-reactive groups are involved in the protonation-deprotonation reaction.

When enzyme is preincubated with pyridoxal 5-phosphate in the dark and thereafter transferred to the test medium with no pyridoxal 5-phosphate

(diluted 1:200), the initial activity is low but increases as a function of time (Fig. 3), i.e., the inhibition by pyridoxal 5-phosphate is reversible. In the experiments shown in Fig. 3 the enzyme was preincubated with 0.4 mM pyridoxal 5-phosphate for 30 min at 22°C, pH 7.4 in the presence of 150 mM Na^+. The reversible inhibition by pyridoxal 5-phosphate can be made irreversible by terminating the preincubation by adding $NaBH_4$ (Fig. 3).

At 22°C, pH 7.4 and with a preincubation time of 30 min in the dark, the irreversible inhibition of the ATPase and of the *p*-nitrophenylphosphatase depends on the concentration of pyridoxal 5-phosphate and on the presence of 150 mM Na^+ and 150 mM K^+, respectively, as shown in Fig. 4. The ATPase activity is more sensitive to treatment with pyridoxal 5-phosphate than the *p*-nitrophenylphosphatase activity. 150 mM K^+ in the preincubation medium gives a certain protection relative to 150 mM Na^+, especially on the inhibition of the *p*-nitrophenylphosphatase activity.

With 0.4 mM pyridoxal 5-phosphate at 22°C, pH 7.4, the inhibition of the ATPase activity in the presence of 150 mM $Na^+ \pm 3$ mM ATP, of 150 mM $K^+ \pm 3$ mM ATP and of 3 mM ATP, respectively, reaches equilibrium after 20–30 min of preincubation (Fig. 5). ATP protects partly against inactivation in the presence both of Na^+ and of K^+.

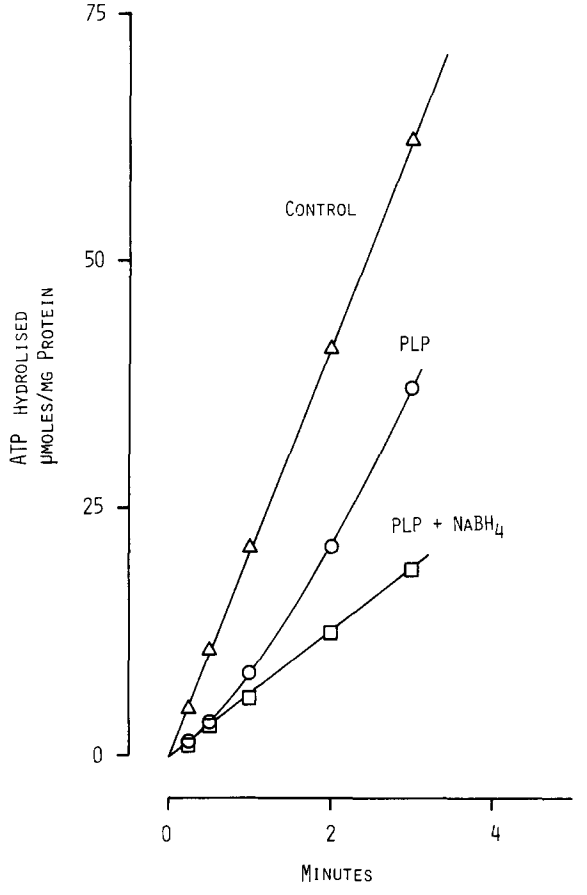


Fig. 3. ATP hydrolysis as a function of time of enzyme which has been preincubated for 30 min in a 30 mM *N*-ethylmorphine-HCl buffer, pH 7.4, at 22°C without (△) and with 0.4 mM pyrodoxal 5-phosphate (PLP) + 150 mM Na^+ (○) and with 0.4 mM pyridoxal 5-phosphate + 150 mM Na^+ but with addition of $NaBH_4$ after the end of preincubation (□). The activity was tested with 3 mM ATP, 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+} in a 30 mM histidine-HCl buffer, pH 7.4, 37°C. The enzyme was diluted 1:200 by transfer from the preincubation to the test medium.

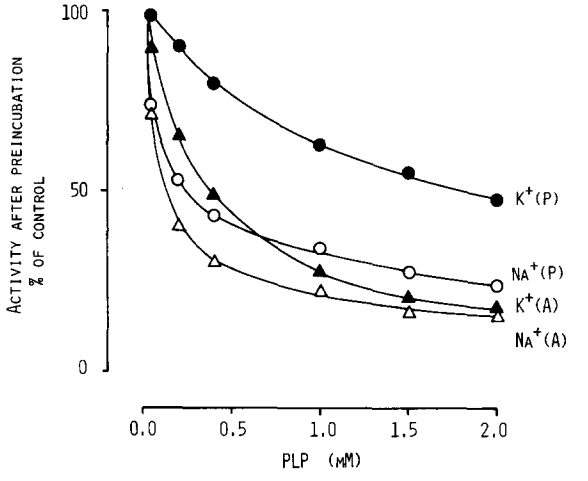


Fig. 4. The ATPase (△, ▲) and the *p*-nitrophenylphosphatase (○, ●) activity after preincubation of the enzyme with pyrodoxal 5-phosphate (PLP). The enzyme was preincubated with the concentration of pyridoxal 5-phosphate given in the figure in a 30 mM *N*-ethylmorpholine-HCl buffer (pH 7.4) for 30 min at 22°C with K^+ 150 mM (▲, ●) and with Na^+ 150 mM (△, ○), respectively. After preincubation, 5-fold excess of $NaBH_4$ was added (two drops of octanol to avoid foaming) and the enzyme was washed three times by centrifugation in a 30 mM histidine-HCl buffer, pH 6.8, at 2°C and finally resuspended in the same buffer but with 25% glycerol. The ATPase activity (△, ▲) was measured with 3 mM ATP, 20 mM K^+, 130 mM Na^+, 4 mM Mg^{2+} in a 30 mM histidine-HCl buffer (pH 7.4) 37°C. The *p*-nitrophenylphosphatase activity (○, ●) was measured with 10 mM *p*-nitrophenyl phosphate, 20 mM Mg^{2+}, 150 mM K^+ in a 30 mM histidine-HCl buffer (pH 7.4) 37°C.

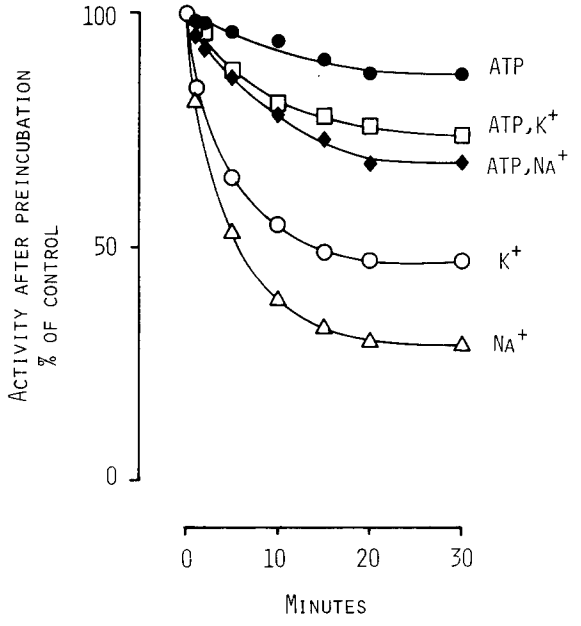


Fig. 5. The ATPase activity after different times of preincubation with pyridoxal 5-phosphate. The enzyme was preincubated in a 30 mM *N*-ethylmorpholine-HCl buffer at pH 7.4, 22°C with 10 mM *trans*-1,2-diaminocyclohexane tetraacetic acid (CDTA), 0.4 mM pyridoxal 5-phosphate and with 3 mM ATP (●), 150 mM K^+ (○), 150 mM Na^+ (△), 150 mM K^+ +3 mM ATP (□) and with 150 mM Na^+ +3 mM ATP (◆), respectively, for the times shown on the figure. After preincubation the enzyme was treated and tested for ATPase activity as given in Fig. 4.

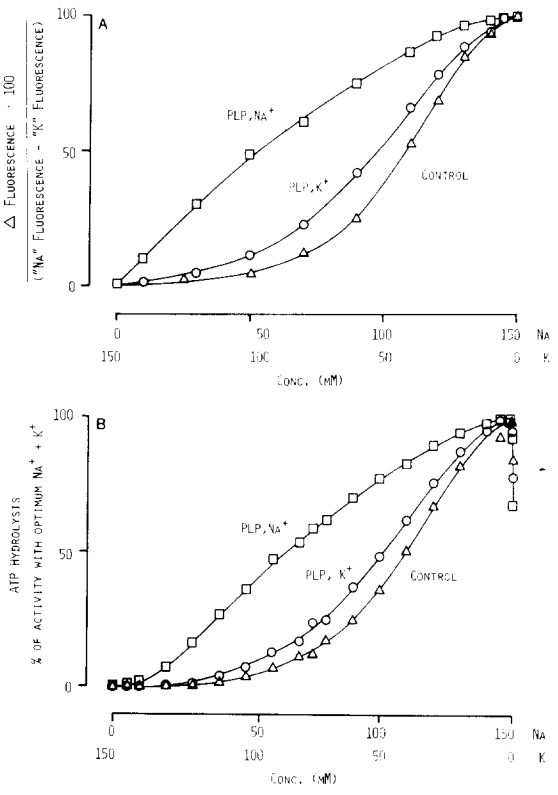


Fig. 6. (A) The effect of different Na^+/K^+ ratios ($Na^+ + K^+ = 150$ mM) on the fluorescence of eosin in the presence of enzyme which has been preincubated with 0.4 mM pyridoxal 5-phosphate (PLP) for 30 min at 22°C (pH 7.4) in the presence of 150 mM Na^+ (□) and of 150 mM K^+ (○), respectively, and of control enzyme (△). 30 mM histine-HCl (pH 7.4) 22°C, 0.1 μM eosin. (B) Activation by $Na^+ + K^+$ ($Na^+ + K^+ = 150$ mM) of the hydrolysis of ATP of the same three enzyme preparations as in (A) (symbols as in (A)). ATP concentration was 0.1 μM, 50 μM Mg^{2+}, 30 mM histidine-HCl buffer (pH 7.4), 37°C.

In the following experiments with pyridoxal 5-phosphate the enzyme has been preincubated with 0.4 mM pyridoxal 5-phosphate and the ligands shown on the figures at 22°C, pH 7.4, for 30 min in the dark, this being followed by addition of 5 mM $NaBH_4$ and three washes by centrifugation.

Modification in presence of Na^+ and of K^+

The Na^+ vs. K^+ titration curve for enzyme which has been preincubated with pyridoxal 5-phosphate in the presence of 150 mM Na^+ is shifted towards a lower Na^+ concentration at pH 7.4, just as was observed for control enzyme by an increase in pH. This is shown for equilibrium conditions in Fig. 6A. With a given amount of enzyme, the difference in eosin fluorescence in the presence of Na^+ and of K^+, respectively, which is used to monitor the change in transformation from the one form to the other, is lower for the enzyme which has been reacted with pyridoxal 5-phosphate than for the control enzyme and the decrease is proportional to the decrease in activity.

The shift towards the lower Na^+ concentration, the 'pH effect' of modification by pyridoxal 5-phosphate, is also seen under steady-state conditions when the hydrolysis of non-saturating concentrations of ATP is used to test the effect (Fig. 6B). This indicates that the enzyme preparation after the modification is not a mixture of modified inactive and of normal active enzyme, but it is the modified enzyme which has reduced activity.

An increase in the concentration of ATP which

shifts the control Na^+ vs. K^+ titration curve towards lower Na^+ values for Na^+ activation of hydrolysis (Fig. 1C) also does this with enzyme which has been modified by pyridoxal 5-phosphate. This is shown for enzyme modified with 0.4 mM pyridoxal 5-phosphate in the presence of 150 mM Na^+ in Fig. 7, which shows the Na^+ concentration for half maximum Na^+ activation ($K_{0.5}$ for Na^+, $Na^+ + K^+ = 150$ mM) as a function of the ATP concentration. The values shown in Fig. 7A are taken from curves like the curve shown in Fig. 1C for control enzyme and in Fig. 6B for enzyme modified by pyridoxal 5-phosphate in the presence of 150 mM Na^+. For comparison is shown the effect of ATP on $K_{0.5}$ for Na^+ activation of a control enzyme at pH 7.4 and 8.4, respectively, Fig. 7B. The values in Fig. 7B are from another enzyme source, ox brain, and is replotted from Fig. 6 in Ref. 11. A comparison of Figs. 7A and 7B shows that the pyridoxal 5-phosphate-modified enzyme at pH 7.4 from the point of view of ATP effect behaves as a non-modified enzyme at a higher pH.

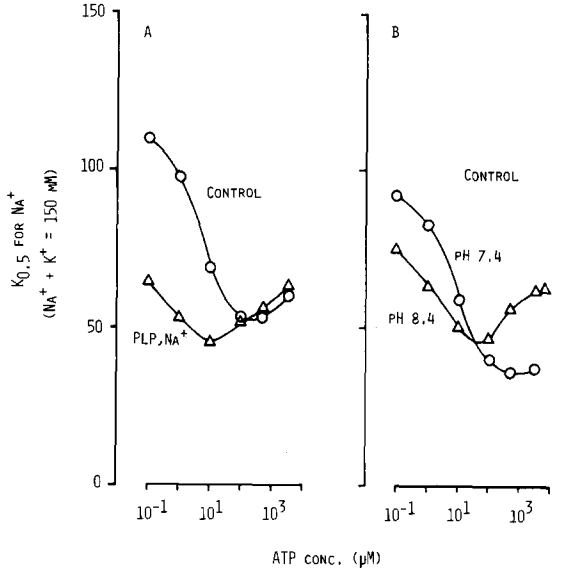


Fig. 7. The concentration of Na^+ for half-maximum activation of hydrolysis ($K_{0.5}$ for Na^+, $Na^+ + K^+ = 150$ mM) at different ATP concentrations. (A) For control enzyme (○), and for enzyme preincubated with 0.4 mM pyridoxal 5-phosphate (PLP) + 150 mM Na^+ for 30 min (pH 7.4), 22°C (△). The $K_{0.5}$ values for Na^+ are read from a series of curves like the curves shown in Figs. 1C and 6B, tested in 30 mM histidine-HCl (pH 7.4), 37°C. (B) For control enzyme of pH 7.4 (○) and 8.4 (△). The values shown in (B) are from an enzyme preparation from ox brain and the values are replotted from Fig. 6 in Ref. 11, 30 mM histidine-HCl at pH 7.4 and 30 mM Tris-HCl at pH 8.4.

Preincubation of enzyme with pyridoxal 5-phosphate in the presence of 150 mM K^+ has only a slight effect on the Na^+ vs. K^+ titration curves both under equilibrium and under steady-state conditions (Figs. 6A and 6B).

ATP effect on modification

The effect on the Na^+ vs. K^+ titration curve of enzyme modified by pyridoxal 5-phosphate in the presence of different ligands does not parallel the effect on the activity. This is seen from Table II which shows the Na^+ concentration ($K_{0.5}$ for Na^+) for half-maximum effect on the equilibrium distribution between the K^+-form and the Na^+-form of the enzyme modified by pyridoxal 5-phosphate in the presence of different combinations of ligands; for comparison, the effect of the modification on activity is shown.

3 mM ATP gives a certain protection against inactivation by 0.4 mM pyridoxal 5-phosphate in the presence of 150 mM Na^+ as well as in the presence of 150 mM K^+. However, in the presence of Na^+, ATP has no effect on the $K_{0.5}$ for

TABLE II

EFFECT OF ENZYME MODIFICATION BY PYRIDOXAL 5-PHOSPHATE ON $K_{0.5}$ AND ACTIVITY

The effect of enzyme modification by pyridoxal 5-phosphate (PLP) in the presence of 150 mM K^+ ±3 mM ATP, of 3 mM ATP and of 150 mM Na^+ ±3 mM ATP on $K_{0.5}$ for Na^+ and on activity. The $K_{0.5}$ values for Na^+ are taken from curves like the curves shown in Fig. 6A. The enzyme activity was tested with 3 mM ATP, 4 mM Mg^{2+}, 130 mM Na^+, 20 mM K^+, 30 mM histidine-HCl (pH 7.4), 37°C. Number of experiments in parentheses.

	$K_{0.5}$ for Na^+ (mM)	Activity (% of control)
Control	108 ± 1 (5)	100
PLP, K^+	98 ± 1 (3)	52 ± 1.2 (4)
PLP, ATP	80 ± 2 (3)	87 ± 1.8 (3)
PLP, K^+, ATP	58 ± 0.2 (3)	72 ± 1.7 (3)
PLP, Na^+	49 ± 2 (6)	31 ± 0.6 (9)
PLP, Na^+, ATP	52 ± 1 (3)	69 ± 1.2 (3)

Na^+, while in the presence of K^+ it shifts the titration curve towards a lower $K_{0.5}$ for Na^+. Preincubation with 3 mM ATP without Na^+ or K^+, which gives the lowest decrease in activity, gives a titration curve with a $K_{0.5}$ for Na^+ which is inbetween the one obtained with 150 mM K^+ and that with 150 mM K^+ + 3 mM ATP.

Effect of modification on rates

Modification of enzyme with pyridoxal 5-phosphate in the presence of 150 mM K^+ has no effect on the rate of the transfer from the K^+-form to the Na^+-form, Table III. The slight effect on the titration curves (Figs. 6A and B) can be explained from the effect on the reverse reaction, Table III. For enzyme which has been modified by pyridoxal 5-phosphate in the presence of 150 mM K^+ + 3 mM ATP, of 150 mM Na^+ without and with 3 mM ATP and of 3 mM ATP, there is an effect on the rates similar to the effect of an increase in pH for a non-modified enzyme. The rate of the transformation from the K^+-form to the Na^+-form is increased and measured at 4°C, the rate of the reverse reaction is decreased. The effect on the rate of the transformation from the K^+-form to the Na^+-form is more pronounced than the effect on the rate of the reverse reaction, just as observed for the effect of an increase in pH (cf. Tables I and III). As seen from a comparison of Tables II and III the effect of the pyridoxal 5-phosphate modification on the rates correlated to the effect on the $K_{0.5}$ for Na^+.

Modification of enzyme by 0.4 mM pyridoxal 5-phosphate in the presence of 3 mM ATP, of 150 mM Na^+ ± 3 mM ATP and of 150 mM K^+ + 3 mM ATP has thus an effect on $K_{0.5}$ for Na^+ for activation of hydrolysis and on the rate of transformation between the two forms as is observed by an increase in pH. 150 mM K^+ protects against

TABLE III

THE HALF-TIME, $t_{1/2}$, IN SECONDS FOR THE TRANSFORMATION FROM THE K^+-FORM TO THE Na^+-FORM OF PYRIDOXAL 5-PHOSPHATE-MODIFIED ENZYME (AND VICE VERSA)

Experimental conditions as in Fig. 2 and TABLE I but with 300 μg of the modified enzyme per ml. The enzyme was preincubated for 30 min with the concentrations and combinations of pyridoxal 5-phosphate (PLP) and ligands described in Fig. 5. After preincubation the enzyme was treated as described in Fig. 4.

	$t_{1/2}$ (s)	
	K^+-form→ Na^+-form	Na^+-form→ K^+-form
22°C, pH 7.4		
Control	0.45 ±0.02	
PLP, K^+	0.46 ±0.02	
PLP, ATP	0.37 ±0.01	
PLP, K^+, ATP	0.11 ±0.016	
PLP, Na^+	0.084±0.008	
PLP, Na^+, ATP	0.095±0.002	
4°C, pH 7.4		
Control	3.33 ±0.04	0.096±0.007
PLP, K^+	3.13 ±0.03	0.156±0.007
PLP, ATP	2.08 ±0.04	0.177±0.004
PLP, K^+, ATP	0.47 ±0.02	0.193±0.006
PLP, Na^+	0.33 ±0.02	0.292±0.012
PLP, Na^+, ATP	0.38 ±0.01	0.273±0.007

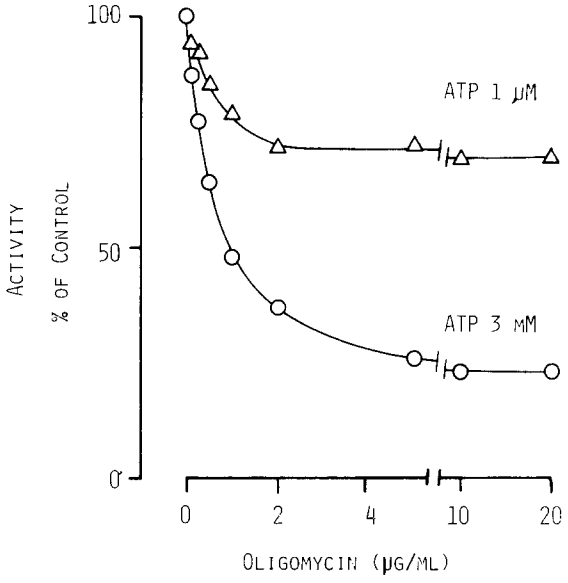


Fig. 8. Effect of oligomycin in the test tube on the ATPase activity in the presence of 1 μM ATP and of 3 mM ATP, respectively. The test solution, 30 mM histidine-HCl buffer (pH 7.4) at 37°C contained 148 mM Na^+, 2 mM K^+ in the experiments with 1 μM ATP and 130 mM Na^+, 20 mM K^+ with 3 mM ATP, which are the combinations of the cations giving maximum activity. The Mg^{2+} concentration was 100 μM and 4 mM. The activity is given as percent of control without oligomycin. Oligomycin was dissolved in absolute alcohol and the amount added per ml test solution was contained in 5 μl alcohol.

the 'pH effect' of the modification by pyridoxal 5-phosphate.

Quantitatively, the modification in the presence of 150 mM K^+ +3 mM ATP has an effect on $K_{0.5}$ for Na^+ under steady-state conditions and on the rates which comes close to the effect of an increase in pH from 7.4 to 8.4. When modified in the presence of 150 mM Na^+ ±3 mM ATP the effect is more pronounced.

Oligomycin

Oligomycin inhibits the enzyme activity but not completely. Maximum effect is obtained with 20 μg/ml in the test tube. The fraction which is inhibited increases with the ATP concentration. With the optimal K^+/Na^+ ratio ($Na^+ + K^+ = 150$

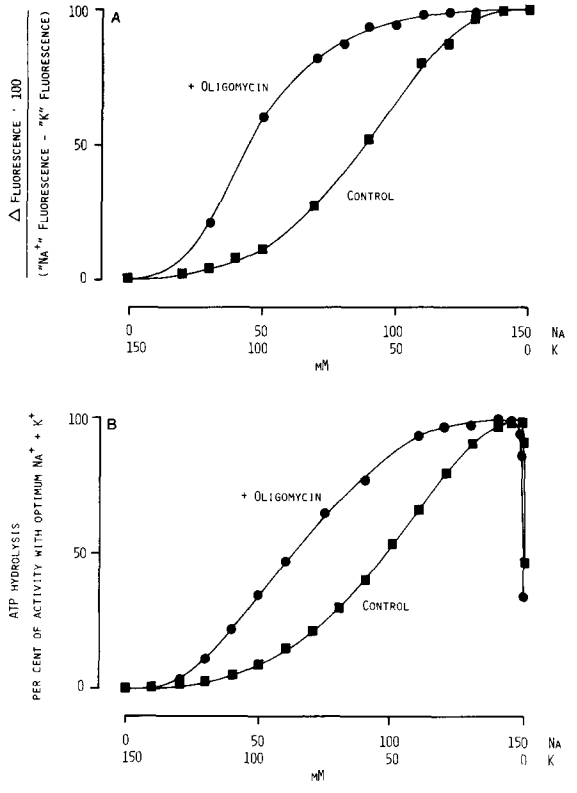


Fig. 9. (A) Na^+ vs. K^+ titration curves ($Na^+ + K^+ = 150$ mM) of the eosin fluorescence in the presence of enzyme without (■) and with 20 μg oligomycin/ml (●). 100 μg enzyme/ml, 30 mM histidine-HCl buffer (pH 7.4) 22°C, 0.1 μM eosin Y. (B) Activation by $Na^+ + K^+$ ($Na^+ + K^+ = 150$ mM) of the hydrolysis of ATP without (■) and with 20 μg oligomycin/ml in the test medium (●). The ATP concentration was 1 μM, 100 μM Mg^{2+}, 30 mM histidine-HCl (pH 7.4), 37°C.

mM) for hydrolysis it is about 30% with 1 μM ATP and about 80% with 3 mM ATP (Fig. 8).

20 μg oligomycin per ml shifts the equilibrium distribution between the K^+-form and the Na^+-form towards the Na^+-form (Fig. 9A). (Oligomycin has no effect on the affinity of the fluorescence probe eosin.) And it decreases the $K_{0.5}$ for Na^+ for Na^+ activation of hydrolysis with a non-saturating concentration of ATP (shown for 1 μM ATP, Fig. 9B). Oligomycin has thus an effect similar to that observed with an increase in pH, or at a given pH with an increase in the ATP concentration, or by modification with pyridoxal 5-phosphate in the presence of certain ligands.

However, oligomycin has practically no effect on the rate of the transfer from the K^+-form to the Na^+-form of the system, but oligomycin decreases the rate of the transfer from the Na^+-form to the K^+-form. The effect is very pronounced, Table I.

Discussion

pH and ATP effect

An increase in pH turns the $(Na^+ + K^+)$-ATPase into the Na^+-form, while a decrease in pH turns the system into the K^+-form, supporting the view that the Na^+-form is a deprotonated and the K^+-form a protonated form of the system [8,11]. There are two K^+ dissociation constants involved, one with a pK between 5 and 7 and another with a pK between 9 and 10 [8].

There is a similarity between the effect of an increase in pH at a non-saturating concentration of ATP and that of an increase in the ATP concentration at a given pH on the Na^+ vs. K^+ titration curves. There is also a similarity between the effect of ATP [5,6] and that of an increase in pH on the rate of the transfer from the K^+-form to the Na^+-form. It suggests that the binding of ATP to the K^+-form facilitates release of protons, decreases pK values in the system and thereby facilitates the transformation from the K^+-form to the Na^+-form of the system, a Bohr effect.

There is, however, a difference between the effect of an increase in pH and of an increase in the ATP concentration at a given pH. The $K_{0.5}$ for Na^+ decreases continuously when pH is increased and is at pH 9.4 (the highest tested) 9 mM (see

Figs. 8 and 10 in Ref. 8). On the other hand, when ATP is increased the effect on $K_{0.5}$ for Na^+ levels off or is replaced by an increase in $K_{0.5}$ at the higher ATP concentrations (Figs. 7A and 7B). Also, the minimum value for $K_{0.5}$ for Na^+ is higher than the values obtained by the pH increase; at pH 7.4, 48 mM with rectal gland enzyme and 37 mM with ox brain enzyme (Figs. 7A and 7B). The effect of ATP on $K_{0.5}$ for Na^+ is thus the result of two opposing effects: one which decreases $K_{0.5}$ for Na^+ like a deprotonation of the system and with a high affinity for ATP; another which increases $K_{0.5}$ for Na^+ but with a low affinity for ATP. Such a double effect seems to require a reaction with two ATP molecules, either simultaneously or consecutively.

The low affinity effect of ATP becomes more pronounced when pH is increased from 7.4 to 8.4 or when the enzyme is modified by pyridoxal 5-phosphate in the presence of Na^+. This indicates (1) that also the low-affinity ATP effect is related to a deprotonation and (2) that in the presence of ATP the deprotonation of certain groups on the system no longer give a decrease but an increase in $K_{0.5}$ for Na^+.

This effect of ATP is observed under ATP hydrolyzing, steady-state conditions.

The present and previous experiments [8,11] show that the affinity for ATP for the effect on the transformation from the K^+-form to the Na^+-form ($K_{0.5}$ for Na^+) is higher than for ATP for the hydrolysis in the presence of $Na^+ + K^+$. An explanation of the double effect of ATP on $K_{0.5}$ for Na^+ may then be that at the low ATP concentration it is not the transformation of the K^+-form to the Na^+-form which is rate-limiting, but a following step which is related to the hydrolysis of ATP. In contrast, at the higher concentration of ATP it is the transformation from the K^+-form to the Na^+-form which becomes rate-limiting and not the hydrolysis step. This is in disagreement with the view that it is the requirement for ATP for the increase in rate of the transformation from the K^+-form to the Na^+-form which determines the K_m for ATP as substrate [5–7] but in agreement with the view that the K_m for ATP is determined by the requirement for MgATP for a translocation step which follows the ATP supported transformation from the K^+-form to the Na^+-form [8–11].

But before investing too much effort in trying to explain the double effect of ATP it is necessary to see if this effect is also observed under ATP non-hydrolyzing conditions. Unfortunately this cannot be done with the fluorescence method used in the present experiments.

Modification with pyridoxal 5-phosphate

The effect of a modification of the enzyme with pyridoxal 5-phosphate which leads to an enzyme which behaves as control enzyme but at a higher pH shows that pyridoxal 5-phosphate-reactive groups (amino groups) are involved in the deprotonation-protonation reaction which leads to the transformation between the two enzymes forms.

There must be at least two different pyridoxal 5-phosphate-reactive groups on the enzyme. ATP protects against the decrease in enzyme activity which follows from the modification by pyridoxal 5-phosphate both with Na^+ and with K^+ in the preincubation medium. But ATP in the preincubation medium has no effect on the 'pH effect' when the enzyme is modified in the presence of Na^+, and increases the effect when it is modified in the presence of K^+. However, enzyme modified in the presence of Na^+ but without ATP still responds to an increase in the ATP concentration in the test medium by a decrease in $K_{0.5}$ for Na^+. It means that the reaction with pyridoxal 5-phosphate which leads to a decrease in activity and which can be prevented by preincubation in the presence of ATP does not block the ATP site. It suggests that the ATP-sensitive pyridoxal 5-phosphate-reactive groups are not on the ATP site; or that there are two different ATP sites, one which has pyridoxal 5-phosphate-reactive groups and another which has not. It indicates that the 'pH effect' of pyridoxal 5-phosphate is not due indirectly to an 'ATP effect' of pyridoxal 5-phosphate on an ATP site but to an effect of a reaction of pyridoxal 5-phosphate with groups (amino groups) which are involved more directly in the protonation-deprotonation reaction.

Pyridoxal 5-phosphate reacts with ϵ-amino groups on lysine [17] i.e., the groups with the higher pK value. However, the effect on $K_{0.5}$ for Na^+ is seen at a pH which is at least two units lower than the pK value for these groups. It means either that the pyridoxal 5-phosphate effect is not

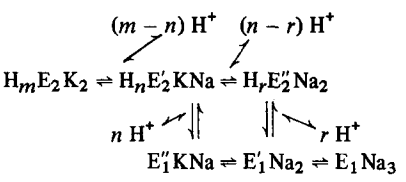
on the epsilon amino groups. Or that reaction with these groups has an effect on the dissociation of the groups with the lower pK, which are involved in the protonation-deprotonation reaction of importance for the transformation between the two forms. In order to solve this problem it is necessary to know the kind of groups pyridoxal 5-phosphate reacts with and also the interplay between these and other groups on the system.

With K^+ in the preincubation medium, i.e., with the enzyme molecules on the protonated K^+-form, the amino groups which are of importance for the pH effect on the conformation are 'hidden' for reaction with pyridoxal 5-phosphate. ATP, which in the presence of K^+ turns the molecules to the Na^+-form [4], exposes the groups, as does preincubation in the presence of Na^+ and of Na^+ + ATP. However, from the point of view of ligand effect on the reactivity towards pyridoxal 5-phosphate, the conformation in the presence of K^+ + ATP is not identical to the Na^+-form. Neither is the conformation in the presence of ATP alone identical to the Na^+-form, suggesting that the transformation from the K^+-form to the Na^+-form is not an either/or situation.

Interpretation

A proton effect on the conformation and with the involved amino groups 'hidden' when protonated (the K^+-form) suggests that the protonated amino groups take part in salt bridge formation inbetween and inside the polypeptide chains, a hemoglobin-like situation [18]. The deprotonated, Na^+-form (E_1) is then the form with the low degree of salt bridge formation, i.e., the relaxed, R-structure in the notation by Monod et al. [19] with a high affinity for Na^+, and, as suggested from the experiments on the effect of the concentration of K^+ on the rate of the transformation from the Na^+-form to the K^+-form [5], with a low affinity for K^+. The protonated K^+-form (E_2) is the form with the high degree of salt bridge formation, the tense, T-structure, with a high affinity for K^+ and low affinity for Na^+.

The cation effect which leads to the conformational change is on the internal sites of the system [8,20]. With either two potassium or three sodium ions bound [21].



The successive binding of Na^+ to E_2 leads to weakening and breaking of the salt bridges, release of protons, and to an increased likelihood of conversion from E_2 to E_1. E_2Na_3 and E_1K_2 are too unstable to exist in more than minute amounts. ATP facilitates the deprotonation by decreasing pK values, i.e., facilitates breaking of the salt bridges and thereby the transformation from the E_2 to the E_1 form.

It is the Na^+-form (E_1) with Na^+ bound which has catalytic activity, and it would be of interest to know whether the H^+ released from ATP when it is hydrolyzed by this form takes part in the reprotonation of the deprotonated E_1-form and thereby increases the rate of the transfer from the Na^+-form (E_1) to the K^+-form (E_2).

Oligomycin

Oligomycin has a 'pH effect' on the distribution between the K^+-form and the Na^+-form under both equilibrium and steady-state conditions. However, oligomycin has no effect on the rate of the transfer from the K^+-form to the Na^+-form but, rather, on the reverse reaction, in agreement with observations by Karlish et al. [5]. The effect of oligomycin can thus not be due to a facilitation of a deprotonation, but oligomycin may in some way interfere with (prevent) a protonation process, suggesting that oligomycin reacts only with the system in the Na^+-form. Oligomycin, which is known to block the E_1-P to E_2-P reaction [22], thus also interferes with the reaction between the non-phosphorylated forms, decreasing the rate of the E_1 to the E_2 transformation, with no effect on the rate of the transformation from E_2 to E_1, and thereby shifts the equilibrium towards the E_1 form.

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