Biochimica et Biophysica Acta 819 (1985) 119–130
Elsevier

BBA 72716

Modification of the $(Na^+ + K^+)$-ATPase with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and with diethylpyrocarbonate. Effect on the conformational transition

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(Received May 7th, 1985)

Key words: $(Na^+ + K^+)$-ATPase; Chemical modification; Conformational transition; (*S. acanthias* rectal gland)

Modification of carboxyl groups of the $(Na^+ + K^+)$-ATPase with the water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide or of amino groups, histidine or alpha-amino groups, with diethylpyrocarbonate gives a pronounced decrease in apparent affinity for K^+ for transition from the Na^+-form to the K^+-form at a given Na^+ concentration. The fluorescence of eosin is used to monitor the conformational transitions. The decrease in apparent affinity for K^+ is due to an increase in the rate of the conformational transition from E_2 with K^+ occluded, $E_2(K_m^+)$, to E_2 with K^+ non-occluded, $E_2K_m^+$, and to a lesser degree to a decrease in the rate of the reverse reaction, while there is little or no effect on the transition between E_2 in the absence of K^+ and $E_1Na_n^+$. The conformational transition between $E_2(K_m^+)$ and $E_2K_m^+$ is thus much more sensitive to a change in the tertiary and/or quaternary structure than the conformational transition between E_2 and $E_1Na_n^+$. The pK values for the carboxyl groups are lower with the enzyme in the K^+-form than in the Na^+-form; in the Na^+-form it seems to be lower than the lowest tested, pH 5.5. The diethylpyrocarbonate reactive amino group has a higher pK with the enzyme in the K^+-form, about 8.0, than in the Na^+-form, about 7.5. Plots of log $K_{0.5}$ for K^+ for reversal of the effect of Na^+ on the conformation vs. log$[Na^+]$ at a given pH, and vs. pH (from 6.8 to 8.0) at a given Na^+ concentration give straight lines, and the slope suggests that 4 Na^+ on E_1, $E_1Na_4^+$, is replaced by 2 K^+ + 1 H^+ on E_2, $E_2(K_2^+)H^+$. Modification of the amino groups with diethylpyrocarbonate shifts the log K^+ vs. pH plot towards a higher K^+ value at a given pH, but the slope is as for control enzyme, while the log K^+ vs. log$[Na^+]$ plot becomes non-linear. None of the plots are linear after modification of the carboxyl groups with the carbodiimide.

Introduction

The transition from the Na^+-form to the K^+-form of the $(Na^+ + K^+)$-ATPase is accompanied by a protonation, a Bohr effect [1,2].

The effect of pH on the reactivity of the enzyme towards pyridoxal 5-phosphate shows that there are two sets of amino groups, which take part in the protonation. The apparent pK of the one is increased from about 7.4 to about 8.0 and of the other from about 7.7 to 9.5–10, when the enzyme shifts from the Na^+-form to the K^+-form [3].

However, the apparent pK for the proton effect on the conformation increases from about 5.5 in the presence of a high Na^+, low K^+ concentration (140 mM Na^+, 10 mM K^+) to about 9.0 in the presence of a low Na^+, high K^+ concentration (25 mM Na^+, 125 mM K^+) [2], suggesting a larger difference in apparent pK with the enzyme in the

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DEP, diethylpyrocarbonate; Mes, 4-morpholineethanesulfonic acid.

Na^+-form and in the K^+-form, respectively, than the 0.6 to 2–2.5 units, which can be observed from the reactivity towards pyridoxal 5-phosphate [3]. This shows that there must be other than the pyridoxal 5-phosphate reactive groups involved in the protonation, which accompanies the transition from the Na^+-form to the K^+-form.

The present paper deals with the effect on the equilibrium and the effect on rate of the transition, between the Na^+-form and the K^+-form, of a modification of the enzyme with the water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and with diethylpyrocarbonate (DEP). The carbodimide reacts with carboxyl groups [4], and diethylpyrocarbonate is known to react with histidine [5].

Methods

Activity. The enzyme is prepared from rectal glands of *Squalus acanthias* and tested as previously described [6]. The specific ATPase activity of the preparations used ranges from 1109 to 1357 μmol P_i/mg protein per h, and the *p*-nitrophenylphosphatase activity from 187 to 205 μmol *p*-nitrophenol/mg protein per h. Protein is determined by the method of Lowry et al. [7] with serum albumin as a standard.

[^{32}P]ATP is used as substrate and the ATPase activity is measured from the release of $^{32}P_i$ which is determined by the method of Lindberg and Ernster [8].

Modification. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride is from Pierce and diethylpyrocarbonate from Sigma.

Modification with the carbodiimide is performed in a 20 mM buffer of a mixture of 20 mM morpholineethanesulfonic acid (Mes) and 20 mM Tris base at 22°C at the pH shown in the figures. The modification is performed with the enzyme in the Na^+-form (150 mM Na^+) or in the K^+-form (150 mM K^+). The reaction is stopped by addition of Tris EDTA to a final concentration of 10 mM, which adjusts the pH to 7.6.

Modification with diethylpyrocarbonate is performed in a 30 mM phosphate buffer with the enzyme in the Na^+-form (150 mM Na^+) or in the K^+-form (150 mM K^+) at 22°C and at the pH shown in the figures. Diethylpyrocarbonate is dissolved in methanol, and 5 μl of the solution is added to 1 ml of the reaction medium. The reaction is stopped by addition of 20 mM histidine-HCl to a final pH of 7.4.

After the preincubation the enzyme is washed free of surplus of the modifying agent and of the ligands by three times centrifugation and resuspension in 20 mM histidine-HCl (pH 6.8) with 25% glycerol. After the final centrifugation the enzyme is stored at −20°C.

Conformational transition. The effect of Na^+ and of K^+ on the transition between the E_1 and the E_2 conformation of the enzyme is monitored by the change in fluorescence of 0.1 μM eosin [9]. The notation E_1 is used for the conformation of the enzyme, which binds eosin with a high affinity; the binding leads to an increase in fluorescence. E_2 is used for the conformation, which binds eosin with a low affinity. In a 20 mM histidine-HCl buffer at pH 7.4 the enzyme is in the E_2 conformation, and this is not due to an effect of histidine or to a contamination by K^+ [10].

The effect of the modification on the apparent affinity for Na^+ is tested by measuring the concentrations of Na^+ necessary for half maximum effect on the transition from E_2 to E_1 in the absence of K^+ ($K_{0.5}$ for Na^+). The $E_1Na_n^+$ conformation (n is a number), is also denoted the Na^+-form.

Binding of K^+ to E_2, $E_2K_m^+$ (m is a number), leads to an occlusion of K^+, to $E_2(K_m^+)$ [11,12]. As the equilibrium between E_1 and E_2 is poised towards E_2 in the absence of K^+, the addition of K^+ with the following transition to $E_2(K_m^+)$ gives no change in the fluorescence of eosin. In order to test the effect of the modification on the apparent affinity for K^+, the enzyme is turned on the E_1-form by the addition of 30 mM Na^+, which gives maximal Na^+ effect, and the concentration of K^+ necessary for half maximal reversal of the effect of Na^+ is measured, $K_{0.5\ (Na\ =30\ mM)}$ for K^+. This means that the effect of the modification on the apparent affinity for K^+ is measured relative to the effect on the apparent affinity for Na^+. K^+-form is the notation used for the E_2 conformation seen in the presence of K^+.

Modification of the enzyme with the carbodiimide or with diethylpyrocarbonate which decreases the activity (see below) has no effect on the

size of the fluorescence response.

The effect of the modification on the apparent affinity for K^+ relative to the apparent affinity for Na^+ is also measured under conditions with the ionic strength kept constant. As there is no inert cation which can replace Na^+ or K^+ this is done by measuring, with $Na^+ + K^+$ kept constant at 150 mM, the $Na^+ : K^+$ ratio which gives half maximum increase in the fluorescence, when the enzyme is titrated from the conformation seen in the presence of 150 mM K^+ to the conformation seen in the presence of 150 mM Na^+. This value is denoted $K_{0.5}$ for the $Na^+ : K^+$ ratio.

The rate of transition in between the different conformations is measured with a stop-flow apparatus connected to a Perkin-Elmer MPF 44 spectrofluorometer; data are collected with a Datalab DL 901 transient recorder interfaced to a HP 85 micro computer as previously described [10].

Results

Modification with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Effect on catalytic activity. Preincubation of the $(Na^+ + K^+)$-ATPase with 10 mM of the carbodiimide for 30 min at 22°C decreases the ATPase as well as the *p*-nitrophenylphosphatase activity. The inhibitory effect of the carbodiimide increases by a decrease in pH in the preincubation medium (Figs. 1A and 1B). pK is about 6.5 for the effect on the ATPase and on the *p*-nitrophenylphosphatase activity with enzyme modified in the Na^+-form. With enzyme modified in the K^+-form the pK is lower, about 5.5, for the effect on the *p*-nitrophenylphosphatase activity but only slightly lower, 6.3, for the effect on the ATPase activity.

The time-course for the inactivation with 10 mM of the carbodiimide at pH 6.1 in 150 mM Na^+ at 22°C is shown in Fig. 2.

Effect of modification on equilibrium distribution between the conformations. The enzyme is reacted in the Na^+-form (150 mM Na^+) and in the K^+-form (150 mM K^+), respectively, with 10 mM of the carbodiimide for 30 min at 22°C, at the pH values shown in Fig. 3. After the enzyme is washed free of the modifying reagents and the ligands, the effect of the modification is tested by measuring

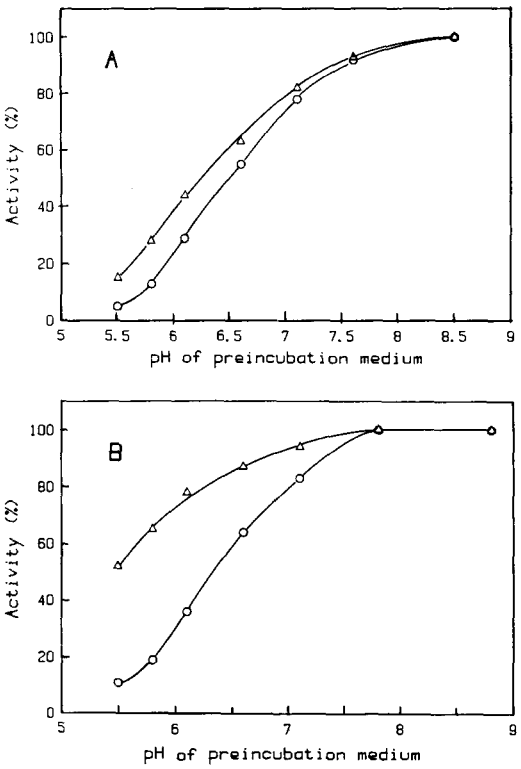

Fig. 1. The effect on the ATPase (A) and on the *p*-nitrophenylphosphatase activity (B) of preincubation of the $(Na^+ + K^+)$-ATPase in the Na^+-form (150 mM Na^+) (○) and in the K^+-form (150 mM K^+) (△), respectively, with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The enzyme is preincubated with 10 mM of the carbodiimide for 30 min at 22°C in a 20 mM Mes-Tris buffer at the pH values shown on the abscissa. The activity is measured in a 30 mM histidine-HCl buffer (pH 7.4), 37°C with 3 mM ATP, 4 mM Mg^{2+}, 130 mM Na^+, 20 mM K^+ for the ATPase activity and 10 mM *p*-nitrophenyl phosphate, 20 mM Mg^{2+}, 150 mM K^+ for the *p*-nitrophenylphosphatase activity. The ouabain insensitive activity serves as a blank. The activity is given in percent of the activity of control enzyme preincubated without EDC.

$K_{0.5}$ for the $Na^+ : K^+$ ratio with $Na^+ + K^+ = 150$ mM, i.e. the $Na^+ : K^+$ ratio which gives half maximum effect on the transition from the conformation with 150 mM K^+ to the conformation with 150 mM Na^+. $K_{0.5}$ for the $Na^+ : K^+$ ratio is measured in a 30 mM histidine-HCl buffer at pH 7.4, 22°C.

Modification with the carbodiimide of the enzyme in the Na^+-form, leads to a decrease in $K_{0.5}$ for the $Na^+ : K^+$ ratio and more so the lower the pH is (Fig. 3). At a given $Na^+ : K^+$ ratio the

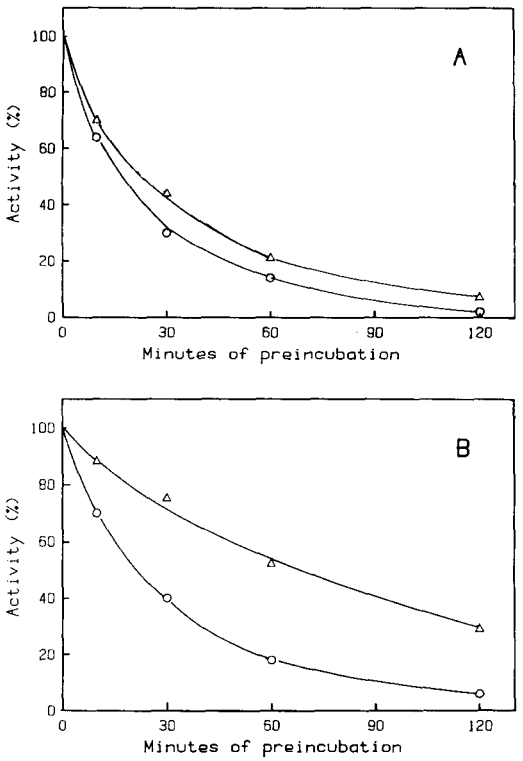

Fig. 2. The time-course of EDC inactivation of the ATPase activity (A) and of the *p*-nitrophenylphosphatase activity (B). The $(Na^+ + K^+)$-ATPase in the Na^+-form (150 mM Na^+) (○) or in the K^+-form (150 mM K^+) (△) is preincubated with 10 mM EDC at 22°C in a 20 mM Mes-Tris buffer at pH 6.1 for the time shown on the abscissa. The activity is measured as in Fig. 1, and is given as percent of the activity of control enzyme preincubated without EDC.

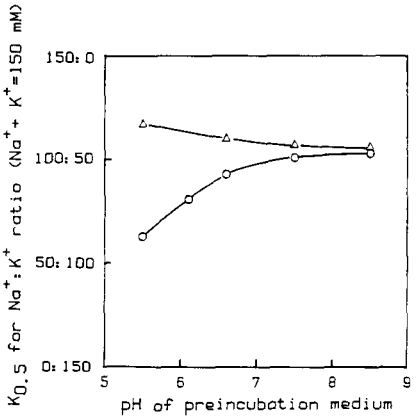

Fig. 3. The $Na^+ : K^+$ ratio for half maximal transition from the conformation in 150 mM K^+ to the conformation in 150 mM Na^+ of $(Na^+ + K^+)$-ATPase modified in the Na^+-form (○) or in the K^+-form (△) with 10 mM EDC for 30 min at 22°C in a 20 mM Mes-Tris buffer at the pH shown on the abscissa. $K_{0.5}$ for the $Na^+ : K^+$ ratio is measured in a 20 mM histidine-HCl buffer (pH 7.4), 22°C with $Na^+ + K^+$ kept constant at 150 mM. The fluorescence of eosin, 0.1 μM, is used to monitor the conformational transition.

modification thus leads to a shift towards the Na^+-form. The apparent pK for the effect seems to be lower than 5.5, which means that it is lower than the pK for the effect on the activity (see Fig. 1). With the enzyme in the K^+-form, the modification with the carbodiimide gives a slight increase in $K_{0.5}$ for the $Na^+ : K^+$ ratio (Fig. 3).

The shift towards the Na^+-form of enzyme modified in the Na^+-form with the carbodiimide is also seen in steady-state experiments where the hydrolysis of ATP is used to monitor the effect. The $Na^+ : K^+$ ratio ($Na^+ + K^+ = 150$ mM) for half maximal Na^+ activation of the hydrolysis of ATP is shifted towards a lower Na^+ value. With enzyme modified in the Na^+-form with 10 mM of the carbodiimide for 30 min at 22°C at pH 6.1 the $Na^+ : K^+$ ratio for half maximal Na^+ activation of the hydrolysis with a suboptimal ATP concentration, 1 μM, is decreased from the control value of 89 : 61 to 59 : 91, and with the optimal ATP concentration, 3 mM, it is decreased from 52 : 98 to 40 : 110 (not shown). This shows that the modification does not lead to a mixture of normal active and of carbodiimide-inactivated enzyme, but that it is the carbodiimide-modified enzyme which has a lower activity.

In another set of experiments an attempt is made to see, whether the effect of the modification on $K_{0.5}$ for the $Na^+ : K^+$ ratio is due to an effect on the apparent affinity for Na^+ and/or on the apparent affinity for K^+. In these experiments the ionic strength cannot be kept constant. The enzyme is modified in the Na^+-form and in the K^+-form, respectively, with 10 mM of the carbodiimide at 22°C, pH 6.1 and for the time shown in Fig. 4.

With no cations in the medium the equilibrium between E_1 and E_2 is poised towards E_2. This means that $K_{0.5}$ for Na^+ for the transition from E_2 to $E_2Na_n^+$ can be titrated. A problem is, however, that buffer cations like Tris and histidine have an 'Na^+-effect' and turn the conformation into the E_1-form [2]. However, at pH 7.4 the effect

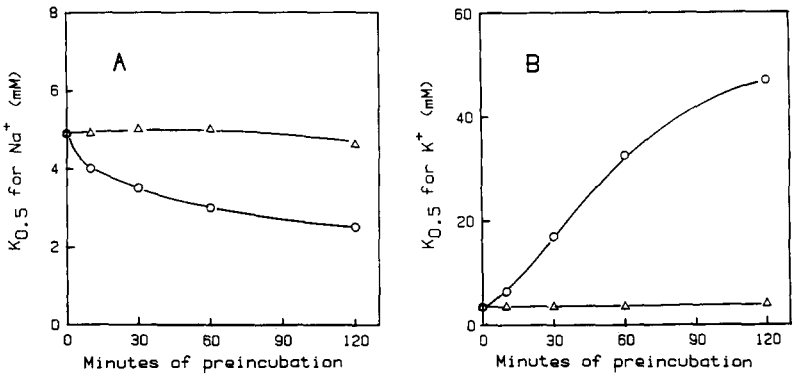

Fig. 4. $K_{0.5}$ for Na^+ for transition from E_2 in the absence of K^+ to $E_1Na_n^+$ (A) and $K_{0.5}$ for K^+ for the reversal of the effect of 30 mM Na^+ on the conformation (B) of (Na^+ + K^+)-ATPase modified with EDC. The enzyme is modified in the Na^+-form (○) or in the K^+-form (△) with 10 mM EDC for 30 minutes at 22°C in a 20 mM Mes-Tris buffer (pH 6.1). $K_{0.5}$ for Na^+ and $K_{0.5}$ for K^+ is measured at 22°C in a 20 mM histidine-HCl buffer (pH 7.4) and the fluorescence of eosin, 0.1 μM, is used to monitor the effect on the conformation.

of histidine is so low, that with 20 mM histidine, practically all the enzyme is in the E_2-form. Under these conditions $K_{0.5}$ for Na^+ for the transition from E_2 to $E_1Na_n^+$ of the non-modified enzyme is 4.9 mM with an eosin concentration of 0.1 μM (Fig. 4A). With the enzyme in the Na^+-form modification with the carbodiimide leads to a slight decrease in $K_{0.5}$ for Na^+, to 2.5 mM after 120 min of modification at the conditions used for the experiment shown in Fig. 4A, suggesting an increase in the apparent affinity for Na^+.

When the enzyme is modified in the K^+-form there is practically no effect on $K_{0.5}$ for Na^+ (Fig. 4A).

$K_{0.5}$ for K^+ for reversal of the effect of 30 mM Na^+ is shown in Fig. 4B. $K_{0.5}$ for K^+ is measured in a 20 mM histidine-HCl buffer at pH 7.4 at 22°C with 30 mM Na^+, and with 0.1 μM eosin to monitor the effect on the conformation.

When the enzyme is modified in the Na^+-form, $K_{0.5}$ for K^+ increases, and after 120 min of modification at pH 6.1, $K_{0.5}$ for K^+ is increased from the control value of 3.5 mM to 47 mM, i.e. about 13-times. $K_{0.5}$ for K^+ is measured with the affinity for Na^+ as a reference. As the apparent affinity for Na^+ is slightly increased by the modification, this by itself gives an increase in $K_{0.5}$ for reversal of the effect of 30 mM Na^+. However, a 13-times increase in $K_{0.5}$ for K^+ is far more than can be explained from the decrease in $K_{0.5}$ for Na^+ from the control value of 4.9 mM to 2.5 mM. The modification of the enzyme in the Na^+-form with the carbodiimide at pH 6.1 thus leads to a moderate increase in apparent affinity for Na^+ for the transition from E_2 to $E_1Na_n^+$ and to a considerable decrease in apparent affinity for K^+ for the transition from $E_1Na_n^+$ to the K^+-form ($E_2K_m^+$ and $E_2(K_m^+)$).

There is no effect on $K_{0.5}$ for K^+ of enzyme modified in the K^+-form, Fig. 4B.

Modification with diethylpyrocarbonate

Effect on catalytic activity. Preincubation of the ($Na^+ + K^+$)-ATPase with 0.2 mM diethylpyrocarbonate for 10 min at 22°C leads to a decrease in ATPase activity and more so the higher pH is (Fig. 5). When the enzyme is modified with diethylpyrocarbonate in 150 mM Na^+, the pK for the effect is about 7.3, while it is slightly higher, about 7.5, when the enzyme is modified in the presence of 150 mM K^+.

Fig. 6 shows the time-course of the inhibition

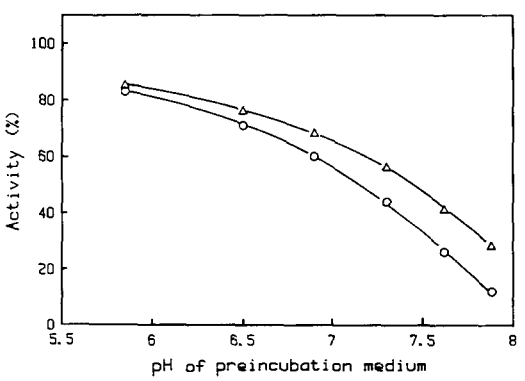

Fig. 5. The ($Na^+ + K^+$)-ATPase activity of enzyme preincubated in the Na^+-form (○) or in the K^+-form (△) with 0.2 mM diethylpyrocarbonate for 10 min at 22°C in a 30 mM phosphate buffer at the pH shown on the abscissa. The activity is measured as in Fig. 1 and is given as percent of the activity of control enzyme preincubated without diethylpyrocarbonate.

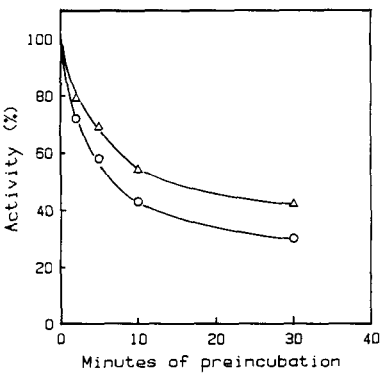

Fig. 6. The time-course of diethylpyrocarbonate inactivation of the $(Na^+ + K^+)$-ATPase activity. The enzyme is preincubated in the Na^+-form (○) or in the K^+-form (△) with 0.2 mM diethylpyrocarbonate at 22°C in a 30 mM phosphate buffer (pH 7.6), for the time shown on the abscissa. The activity is measured and given as in Fig. 5.

by 0.2 mM diethylpyrocarbonate at 22°C pH 7.6 in 150 mM Na^+ and 150 mM K^+, respectively. Diethylpyrocarbonate reacts with water, which explains the levelling off of the effect.

Effect on equilibrium between the conformations. The enzyme is modified with 0.2 mM diethylpyrocarbonate for 10 min at 22°C at the pH value shown in Fig. 7.

$K_{0.5}$ for the $Na^+ : K^+$ ratio decreases when the enzyme is modified in the Na^+-form as well as in the K^+-form, and the effect is more pronounced the higher pH is (Fig. 7). The apparent pK for the reaction with diethylpyrocarbonate is about 7.5 with the enzyme in the Na^+-form while it is about 0.5 units higher with the enzyme in the K^+-form (Fig. 7).

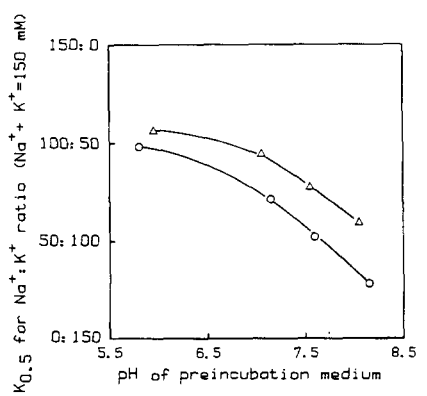

Fig. 7. The $Na^+ : K^+$ ratio for half-maximal transition from the conformation in 150 mM K^+ to the conformation in 150 mM Na^+ of $(Na^+ + K^+)$-ATPase modified in the Na^+-form (○) or in the K^+-form (△) with 0.2 mM diethylpyrocarbonate at 22°C for 10 min in a 30 mM phosphate buffer (pH 7.6). $K_{0.5}$ for the $Na^+ : K^+$ ratio is measured at 22°C in a 20 mM histidine-HCl buffer (pH 7.4), and the fluorescence of eosin, 0.1 μM, is used to monitor the effect on the conformation.

The shift towards the Na^+-form of the diethylpyrocarbonate modified enzyme is also seen in steady-state experiments where the hydrolysis of ATP is used to monitor the effect. With enzyme modified in the Na^+-form with 0.2 mM diethylpyrocarbonate for 10 min at 22°C at pH 7.6 the $Na^+ : K^+$ ratio for half maximal Na^+ activation of hydrolysis with a suboptimal ATP concentration, 1 μM, is decreased from the control value of 94 : 56 to 77 : 73 and with the optimal ATP concentration, 3 mM, it is decreased from 50 : 100 to 41 : 109 (not shown).

In the following experiment the enzyme is modified in the Na^+-form and in the K^+-form, respectively, with 0.2 mM diethylpyrocarbonate at 22°C pH 7.6 and for the time shown in Figs. 8A and 8B. $K_{0.5}$ for Na^+ and $K_{0.5}$ for K^+ for reversal of the effect of 30 mM Na^+ (see Methods) is measured in a 20 mM histidine-HCl buffer at pH 7.4 at 22°C.

With the enzyme modified in the Na^+-form for up to 30 minutes there is no effect of the modification on $K_{0.5}$ for Na^+ for the transition from E_2 to $E_1Na_n^+$ (Fig. 8A). With the enzyme modified in the K^+-form, $K_{0.5}$ for Na^+ is slightly higher, 5.4 mM, than the control value, 4.9 mM (Fig. 8A); this higher value is, however, independent of the time of preincubation.

$K_{0.5}$ for K^+ for reversal of the effect of 30 mM Na^+ increases with the time of preincubation. With the enzyme modified in the Na^+-form, $K_{0.5}$ increases from 3.8 mM to 25 mM after 30 min of reaction with diethylpyrocarbonate (Fig. 8B). With the enzyme modified in the K^+-form, the increase in $K_{0.5}$ for K^+ is less pronounced, from 3.8 mM to about 9 mM after 30 min of preincubation (Fig. 8B). The lower effect with the enzyme in the K^+-form than in the Na^+-form is in agreement with a higher pK for the reaction (cf. Fig. 6). Modification of the enzyme with diethylpyrocarbonate has thus little or no effect on the apparent affinity for Na^+ but decreases the apparent affinity for K^+ for reversal of the effect of Na^+ on the conformation.

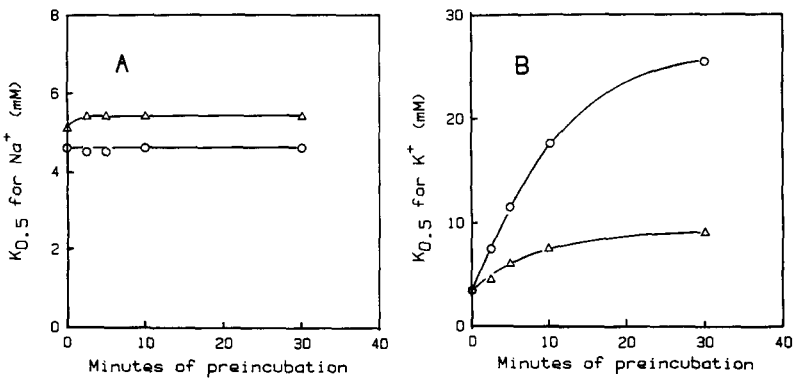

Fig. 8. $K_{0.5}$ for Na^+ for the transition from E_2 in the absence of K^+ to $E_1Na_n^+$ (A) and $K_{0.5}$ for K^+ for reversal of the effect of 30 mM Na^+ on the conformation (B) of diethylpyrocarbonate-modified enzyme. The ($Na^+ + K^+$)-ATPase is modified in the Na^+-form (○) or in the K^+-form (△) with 0.2 mM diethylpyrocarbonate at 22°C for 10 min in a 30 mM phosphate buffer (pH 7.6). The effect on the conformation of Na^+, and of K^+ in the presence of 30 mM Na^+ is measured at 22°C in a 20 mM histidine-HCl buffer (pH 7.4), and the fluorescence of eosin, 0.1 μM, is used to monitor the effect.

Effect of modification on rates of conformational transition

With the enzyme in the Na^+-form modification with 10 mM of the carbodiimide for 30 min at 22°C (pH 6.1) or with 0.2 mM diethylpyrocarbonate for 10 minutes at 22°C (pH 7.6) has no effect on the rate of transition from E_2 in the absence of K^+ to $E_1Na_n^+$ (Table I). As there is no effect on $K_{0.5}$ for Na^+ for the equilibrium distribution between E_2 and $E_1Na_n^+$ of the diethylpyrocarbonate modified enzyme (cf. Fig. 8A), this means that neither is there an effect on the rate of transition from $E_1Na_n^+$ to E_2; this rate cannot be measured with eosin as a probe. With the enzyme in the Na^+-form modification with the carbodiimide as described in Table I gives a slight decrease in $K_{0.5}$ for Na^+ from about 4.9 to about 3.8 (Fig. 4A). With no effect on the rate of transition from E_2 to $E_1Na_n^+$ this means a slight decrease in the rate of the reverse reaction.

The rate of transition from $E_2(K_m^+)$ to $E_1Na_n^+$ is increased by the modification, and there is a less pronounced decrease in the rate of transition from $E_1Na_n^+$ to $E_2(K_m^+)$ (Table I). The shift in the equilibrium distribution between $E_2(K_m^+)$ and $E_1Na_n^+$ towards $E_1Na_n^+$, which follows both from the modification with the carbodiimide, and from the modification with diethylpyrocarbonate, is thus due to an increase in the rate of transition from $E_2(K_m^+)$ to $E_1Na_n^+$, and to a lesser degree due to a

TABLE I

THE EFFECT OF MODIFICATION OF THE ($Na^+ + K^+$)-ATPase WITH 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL)CARBODIIMIDE (EDC) AND WITH DIETHYLPYROCARBONATE (DEP) ON THE RATE OF TRANSITION BETWEEN THE DIFFERENT CONFORMATIONS OF THE ENZYME

The enzyme in the Na^+-form is reacted with 10 mM of EDC at 22°C for 30 min in 20 mM Mes-Tris buffer (pH 6.1) or with 0.2 mM DEP for 10 min at 22°C in 30 mM phosphate buffer (pH 7.6). In the measurements of the rate of the transition from E_2 to E_1, syringe 1 contained enzyme, no K^+, 3 mM K^+ and 10 mM K^+, respectively, and 0.5 μM eosin, 15 mM histidine-HCl, pH 7.2 at 6°C. Syringe 2 contained 0.5 μM eosin, 15 mM histidine-HCl, pH 7.2, 6°C and 100 mM Na^+. In the measurements of the rate of the transition from E_1 to E_2, syringe 1 contained enzyme, 20 mM Na^+, 0.5 μM eosin, 15 mM histidine-HCl, pH 7.2 at 6°C, and syringe 2 contained 40 mM K^+, 0.5 μM eosin and 15 mM histidine-HCl, pH 7.2 at 6°C. The experiments are performed at 6°C. $n = 3$–5.

	$t_{1/2}$ (ms); enzyme		
	control	EDC-modified	DEP-modified
E_2 to E_1			
K^+ 0 mM → Na^+ 100 mM	93 ± 7	93 ± 10	83 ± 5
K^+ 3 mM → Na^+ 100 mM	1069 ± 83	134 ± 2	265 ± 24
K^+ 10 mM → Na^+ 100 mM	861 ± 58	223 ± 25	229 ± 19
E_1 to E_2			
Na^+ 20 mM → K^+ 40 mM	42 ± 6	92 ± 10	88 ± 6

decrease in the rate of the reverse reaction. As there is no effect on the rate of the E_2 to $E_1Na_n^+$ transition in the absence of K^+, and little or no effect on the rate of the reverse reaction this means that the modification leads to an increase in the rate of the $E_2(K_m^+)$ to $E_2K_m^+$ transition, and to a decrease in the rate of the reverse reaction.

Effect of the modification on the $Na^+ : K^+ : H^+$ stoichiometry

The transition from the Na^+-form to the K^+-form of the enzyme is accompanied by a protonation [1,2]. A simple scheme for the reaction is the following

$$E_1Na_n^+ + mK^+ + rH^+ \rightleftharpoons E_2(K_m^+)H_r^+ + nNa^+$$

(m, n and r are numbers)

or

$$\frac{E_1Na_n^+ \cdot [K^+]^m \cdot [H^+]^r}{E_2(K_m^+)H_r \cdot [Na^+]^n} = K$$

with $E_1Na_n^+ = E_2(K_m^+)H_r^+$, taking the log and rearranging *

$$n \log[Na^+] = m \log[K^+] + r \log[H^+] - \log K$$

If this describes the reaction, a plot of $\log[Na^+]$ vs. $\log K_{0.5}$ for K^+ for reversal of the effect of Na^+ on the conformation at a given pH gives a straight line with a slope which is m/n and a plot of $\log K_{0.5}$ for K^+ vs. pH at a given Na^+ concentration gives a straight line with a slope which is m/r.

The enzyme in the Na^+-form is reacted with 10 mM of the carbodiimide for 30 min at 22°C (at pH 6.1) or with 0.2 mM diethylpyrocarbonate for 10 min at 22°C (pH 7.6). $K_{0.5}$ for K^+ for the reversal of the effect of 10, 20, 30, and 50 mM Na^+, respectively, is measured at pH 6.6 and 7.4 for control enzyme, and at pH 7.4 for enzyme modified with the carbodiimide or with diethylpyrocarbonate. The plots of $\log[Na^+]$ vs. $\log K_{0.5}$ for K^+ are shown in Fig. 9. With the same enzyme preparations $K_{0.5}$ for K^+ for reversal of the effect

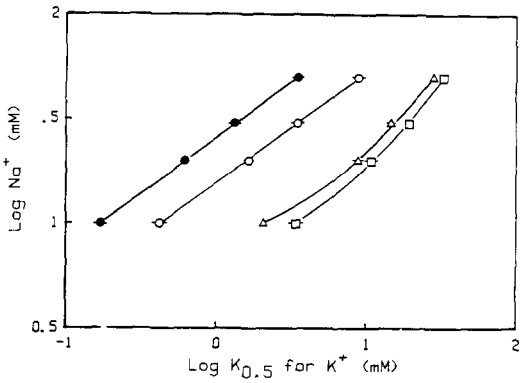

Fig. 9. Plots of $\log[Na^+]$ vs. $\log K_{0.5}$ for K^+ for the reversal of the effect of 10, 20, 30 and 50 mM Na^+, respectively, on the conformation of control enzyme and of enzyme modified with EDC or with diethylpyrocarbonate. The $(Na^+ + K^+)$-ATPase in the Na^+-form is modified with 10 mM EDC at 22°C in a 20 mM Mes-Tris buffer at pH 6.1 or with 0.2 mM diethylpyrocarbonate at 22°C for 10 min in a 30 mM phosphate buffer (pH 7.6). $K_{0.5}$ is measured at pH 6.6 in a 3 mM histidine-HCl buffer for control enzyme (●) and at pH 7.4 in a 3 mM Tris-HCl buffer for control enzyme (○) and for the carbodiimide (□) and the diethylpyrocarbonate-(△)-modified enzyme. 0.1 μM eosin is used to monitor the effect on the conformation.

of 20 mM Na^+ is measured at different pH values. The plots of $\log K_{0.5}$ for K^+ vs. pH are shown in Figs. 10 and 11. In order to minimize the Na^+ effect of the buffer cations, the titration is performed at a buffer concentration of 3 mM, histidine-HCl or Tris-HCl, dependent on the pH.

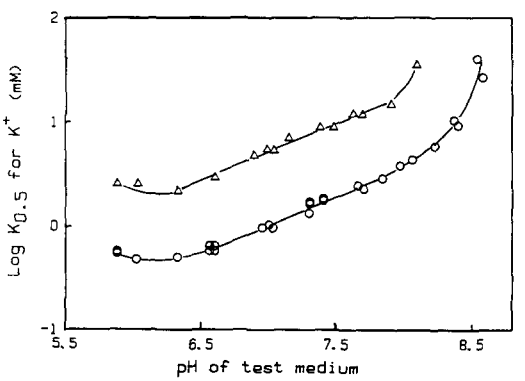

Fig. 10. Plots of log $K_{0.5}$ for K^+ for reversal of the effect of 20 mM Na^+ on the conformation vs. pH of control enzyme (○) and of diethylpyrocarbonate-modified enzyme (△). The $(Na^+ + K^+)$-ATPase in the Na^+-form is modified with 0.2 mM diethylpyrocarbonate at 22°C for 10 min in a 30 mM phosphate buffer (pH 7.6). $K_{0.5}$ for K^+ is measured in a 3 mM histidine-HCl or Tris-HCl buffer and the fluorescence of eosin, 0.1 μM, is used to monitor the effect on the conformation.

* Suggested to me by R.L. Post.

The log[Na^+] versus log $K_{0.5}$ for K^+ plot is linear for control enzyme; a decrease in pH from 7.4 to 6.6 shifts the curve towards a lower K^+ value at a given Na^+ concentration but with no change in the slope (Fig. 9). The correlation coefficient of the determination, r^2, is 0.99 and the slope is 0.52 ± 0.001 ($n = 3$), indicating that the E_2 conformation binds fewer K^+ than the E_1 conformation binds Na^+. The slope value is close to a $Na^+ : K^+$ ratio of 2 : 1.

Modification with the carbodiimide and with diethylpyrocarbonate of the enzyme in the Na^+-form shifts the curve at pH 7.4 towards a higher K^+ value at a given Na^+ concentration. However, the curves are no longer linear, the slope increases with the Na^+ concentration; there is no longer a fixed $Na^+ : K^+$ stoichiometry as for control enzyme (Fig. 9).

The log[K^+] vs. pH plot with 20 mM Na^+ is linear for control enzyme inside the pH interval from about 6.6 to about 8.0 (Fig. 10). The slope is 0.52, suggesting that inside this pH interval 1 H^+ is bound for each 2 K^+. At a pH lower than 6.6 the slope is lower, and seems to be negative at a pH lower than 6.0, indicating that the transition from the Na^+-form to the K^+-form at this lower pH leads to a decrease in protonation, i.e. the Na^+-form is more protonated than the K^+-form. This is the pH at which the protonation of the carboxyl groups are influenced by a change in pH.

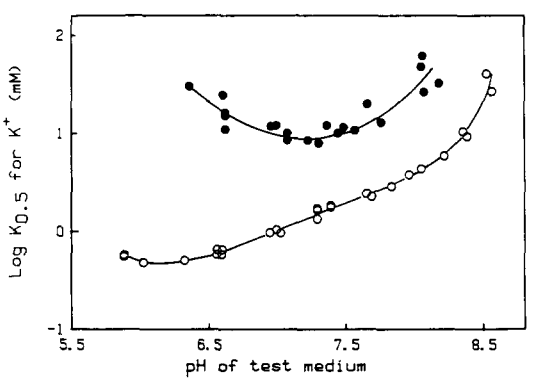

Fig. 11. Plots of log $K_{0.5}$ for K^+ for reversal of the effect of 20 mM Na^+ on the conformation vs. pH of enzyme modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (●). For comparison is shown the curve for control enzyme taken from Fig. 10 (○). The ($Na^+ + K^+$)-ATPase is modified with 10 mM of the carbodiimide at 22°C for 30 min in a 20 mM Mes-Tris buffer (pH 6.1). $K_{0.5}$ for K^+ is measured as in Fig. 10.

At a pH higher than 8, the slope increases with pH.

The log[K^+] vs. pH curve of the diethylpyrocarbonate-modified enzyme is shifted towards a higher K^+ value at a given pH, or towards a lower pH value at a given concentration of K^+, but the pattern is the same as for control enzyme (Fig. 10). The lower apparent affinity for K^+ can thus at a given K^+ concentration be compensated for by a decrease in pH.

With the carbodiimide modified enzyme the part of the curve with the negative slope is shifted upwards towards pH 7.0 (Fig. 11); it is not possible from the present data to tell whether the curve above pH 7.0 is linear or as shown in Fig. 11 is curved with an increasing positive slope.

Discussion

The different pK values for the effect on the ATPase activity, on the *p*-nitrophenylphosphatase activity and on the conformational transition, suggest that a number of carboxyl groups reacts with the carbodiimide, and that it are different carboxyl groups which are of importance for the activities, and for the conformational transition. The carboxylgroups of importance for the activity have a higher pK value with the enzyme in the Na^+-form, than in the K^+-form. The negative slope of the log[K^+] vs. pH curve at a pH below 6.0 suggests that this is also the case for the carboxyl groups involved in the conformational transition. This is in contrast to the diethylpyrocarbonate-reactive amino groups and also to the pyridoxal-5-phosphate-reactive amino groups [3], which have a lower pK with the enzyme in the Na^+-form.

The pH profiles for the effect of the modification with diethylpyrocarbonate on the activity, as well as on the conformational transition, is in agreement with the reaction of diethylpyrocarbonate with amino groups, but the apparent pK is higher than for histidine groups in a water phase. This could be due to an effect of the environment on the histidine groups. Another possibility is that the observed effect is not due to a reaction with histidine groups but with alpha-amino groups. For methodological reasons it is not possible to test whether the reaction with diethylpyrocarbonate leads to an increase in the absorp-

tion at 240 nm, which is an indication of a reaction with histidine groups [5]. Another indication is reversal of the effect of diethylpyrocarbonate by incubation with hydroxylamine [5]. So far no conditions have been found under which hydroxylamine reverses the effect of diethylpyrocarbonate on the activity and on the conformational transition, which may suggest that the observed effect of diethylpyrocarbonate is due to a reaction with alpha-amino groups.

Modification, with the carbodiimide as well as with diethylpyrocarbonate and as shown previously with pyridoxal 5-phosphate [3], has a pronounced effect on the overall equilibrium between the K^+-form and the Na^+-form, shifts the equilibrium towards the Na^+-form at a given $Na^+ : K^+$ ratio. In steady-state experiments this is observed as a decrease in the $Na^+ : K^+$ ratio for half-maximal Na^+ activation of hydrolysis. The effect is mainly due to an increase in the rate of transition from $E_2(K_m^+)$ to $E_2K_m^+$ and to a decrease in the rate of the reverse reaction. Modification with diethylpyrocarbonate has no effect on the E_2 to $E_1Na_n^+$ equilibrium or rate of transition in the absence of K^+. Modification with the carbodiimide gives a slight shift in this equilibrium towards $E_1Na_n^+$, while modification with pyridoxal 5-phosphate gives a slight shift towards E_2 [3]. The major effect of modification with these agents is thus on the conformational transition which leads to the occlusion/deocclusion of K^+; the modification facilitates the deocclusion, the opening of the gate for K^+.

The experiments suggest that the apparent high affinity for K^+, which follows from the conformational transition from $E_2K_m^+$ to $E_2(K_m^+)$ is highly dependent on the tertiary and the quaternary structure of the system, while a change in this structure has little effect on the conformational transition from E_2 in the absence of K^+ to $E_1Na_n^+$. It may however be that the occlusion of Na^+, which follows from the phosphorylation from ATP [13], is more sensitive to a structural modification.

The pH interval for the pH effect on the conformational transition, from pH 5.5 to 9.0 (the limits for the test) [2] can be explained from an involvement of carboxyl groups, of alpha- or histidine-amino groups, and of epsilon-amino groups on lysine. The adaptation of the cation binding sites to Na^+ vs. K^+ leads to a molecular rearrangement, which involves these groups but probably also other groups in the system.

The linear $\log[Na^+]$ vs. $\log[K^+]$ plot with a slope of 0.52 suggests that in the transition from the Na^+-form to the K^+-form 2 Na^+ is replaced by 1 K^+. The linear $\log[K^+]$ vs. pH plot inside pH 6.8–8.0 with a slope of 0.52 suggests that inside this pH interval at each pH the binding of 2 K^+ is accompanied by a binding of 1 H^+ to E_2, suggesting a $Na^+ : K^+ : H^+$ of ratio of 4 : 2 : 1

$$E_1Na_4^+ + 2\,K^+ + 1\,H^+ \rightleftarrows E_2(K_2^+)H^+ + 4\,Na^+$$

This disagrees with the 3 Na^+ to 2 K^+ but agrees with the 2 K^+ to 1 H^+ stoichiometry obtained from a titration of enzyme labelled with fluorescein isothiocyanate (FITC) (Post, R.L., personal communication). It is of interest to know whether this difference is due to the different method used to monitor the effect of the titration. FITC reacts with amino groups probably on lysine [14] an thereby modifies the enzyme, while eosin binds non-covalently to what seems to be the ATP binding site [9].

A pH-independent (from pH 6.8 to 8.0) net uptake of 1 H^+ per 2 K^+ must mean, that there is the same balance between pK increase and pK decrease of groups in the system at each pH value inside this interval when the conformation shifts from the Na^+-form to the K^+-form. This suggests that there is an interaction between the groups which increases their pK, the amino groups, and the groups which decrease their pK, the carboxyl groups, that they are linked by protons, form salt bridges. At the extreme pH values, lower than 6.6 and higher than 8.0, the balance is broken. At a pH lower than 6.6 at which the diethylpyrocarbonate and the pyridoxal 5-phosphate-reactive amino groups are fully protonated with the enzyme in the Na^+-form as well as in the K^+-form, the transition from the Na^+-form to the K^+-form leads to a net release of H^+ probably from the carboxyl groups. At a pH higher than 8.0, at which the carboxyl groups are fully deprotonated with the enzyme in the Na^+-form as well as in the K^+-form, the transition from the Na^+-form to the K^+-form leads to an increased net uptake of H^+ which probably are bound to the amino groups.

But where is the 1 H^+ bound when the con-

formation shifts from the Na^+-form to the K^+-form, inside the pH interval from 6.8 to 8.0? Is it bound to one of the cation binding sites which becomes empty when 4 Na^+ are replaced by 2 K^+? Does the molecular rearrangement when K^+ is bound instead of Na^+, and which can be monitored from the change in pK of the diethylpyrocarbonate-, the pyridoxal-5-phosphate- and the carbodiimide-reactive groups, lead to an increase in pK of one of the cation binding sites to such an affinity for H^+ that H^+ is bound instead of K^+ as well as Na^+? And what happens to the fourth cation binding site, is it eliminated by the conformational transition? Or does the H^+ not occupy a cation binding site but does the binding of H^+ to the system lead to the disappearence of the two binding sites for the cations? Whatever the explanation is it seems to be characteristic for the E_1-form that it has more binding sites for Na^+ than the E_2-form has for K^+, the present experiments suggest a 4 : 2 ratio. This is a higher $Na^+ : K^+$ ratio than the 3 : 2 ratio for the Na^+-K^+ exchange [15]. If the 4 : 2 ratio is correct and not based on too simple a reaction scheme, there is 1 Na^+ bound which is not transported but must go on and off to the cytoplasmic side in the reaction which leads to the translocation of 3 Na^+. The 3 : 2 stoichiometry for the Na^+-K^+ exchange [15] and the electrogenic effect of the exchange [16] shows that the H^+, if it is bound to a cation site cannot be translocated but must go on and off to the *cis* side in the translocation reaction. For a discussion of a possible implication for a transport model of a binding of H^+ together with 2 K^+ see Refs. 10 and 17.

Modification with diethylpyrocarbonate interfers with the $Na^+ : K^+$ but not with the $K^+ : H^+$ stoichiometry, while modification with the carbodiimide interfers with the $Na^+ : K^+$ as well as with the $K^+ : H^+$ stoichiometry.

The present experiments do not tell how many amino groups and carboxyl groups are of importance for the conformational transition, neither do they give the result of the reaction with the carbodiimide. Is it formation of an *O*-acylisourea, or is this rearranged to give an *N*-acylurea, or does it react with a nucleophile inside the system with formation of a covalent bond [4]?

With unmodified ($Na^+ + K^+$)-ATPase, an increase in pH [1,2,3,18] has an effect like the modification with the carbodiimide, with diethylpyrocarbonate and with pyridoxal 5-phosphate [3,18]. The conformation of the enzyme is shifted towards the Na^+-form at a given $Na^+ : K^+$ concentration ratio [1–3] and this is due to an increase in the rate of the transition from the K^+-form to the Na^+-form and to a lesser decrease in the rate of the reverse reaction [18]. At a given pH, an increase in the ATP concentration shifts the conformation towards the Na^+-form at a given $Na^+ : K^+$ concentration ratio [1,2,18] and ATP increases the rate of the transition from the K^+-form to the Na^+-form [19]. This same effect of modification, of an increase in pH and of an increase in the ATP concentration suggests that the binding of ATP to the ATP binding site leads to a molecular rearrangement with a change in the protonation of groups in the system and thereby influences the conformational transition, increases the rate of the transition towards the Na^+-form. The present experiments suggest that the main effect is on the rate of the transition between $E_2(K^+_m)$ and $E_2K^+_m$. Conversely the adaptation of the cation sites to the cations, Na^+ and K^+ leads to a molecular rearrangement which changes the affinity for ATP, high with Na^+ and low with K^+ [20,21]. The quaternary and the tertiary structure of the system links the conformation of the ATP site to the conformation of the cation sites.

Acknowledgements

The author wish to thank Ms. B. Holm and H. Zacharias for excellent technical assistence. The work is supported by the Danish Medical Research Counsil and by 'Ingeborg and Leo Dannin's Foundation for Scientific Research'.

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