Biochimica et Biophysica Acta, 525 (1978) 252—264

BBA 68465

CONFORMATIONAL TRANSITIONS BETWEEN Na^+-BOUND AND K^+-BOUND FORMS OF (Na^+ + K^+)-ATPase, STUDIED WITH FORMYCIN NUCLEOTIDES

S.J.D. KARLISH [a], D.W. YATES [b] and I.M. GLYNN [c]

[a] *Department of Biochemistry, Weizmann Institute, Rehovoth (Israel),* [b] *Department of Biochemistry, University of Bristol, Bristol BS8 1TD, and* [c] *Physiological Laboratory, University of Cambridge, Cambridge, CB2 3EG (U.K.)*

(Received September 22nd, 1977)
(Revised manuscript received February 16th, 1978)

Summary

1. Fluorescence measurements have shown that formycin triphosphate (FTP) or formycin diphosphate (FDP) bound to (Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in Na^+-containing media can be displaced by the following ions (listed in order of effectiveness): Tl^+, K^+, Rb^+, NH_4^+, Cs^+.

2. The differences between the nucleotide affinities displayed by the enzyme in predominantly Na^+ and predominantly K^+ media in the absence of phosphorylation, are thought to reflect changes in enzyme conformation. These changes can therefore be monitored by observing the changes in fluorescence that accompany net binding or net release of formycin nucleotides.

3. The transition from a K^+-bound form ($E_2 \cdot (K)$) to an Na^+-bound form ($E_1 \cdot Na$) is remarkably slow at low nucleotide concentrations, but is accelerated if the nucleotide concentration is increased. This suggests that the binding of nucleotide to a low-affinity site on $E_2 \cdot (K)$ accelerates its conversion to $E_1 \cdot Na$; it supports the hypothesis that during the normal working of the pump, ATP, acting at a low affinity site, accelerates the conversion of dephosphoenzyme, newly formed by K^+-catalysed hydrolysis of E_2P, to a form in which it can be phosphorylated in the presence of Na^+.

4. The rate of the reverse transformation, $E_1 \cdot Na$ to $E_2 \cdot (K)$, varies roughly linearly with the K^+ concentration up to the highest concentration at which the rate can be measured (15 mM). Since much lower concentrations of K^+ are sufficient to displace the equilibrium to the K-form, we suggest that the sequence of events is: (i) combination of K^+ with low affinity (probably internal) binding

---

Abbreviations: FTP, formycin triphosphate; FDP, formycin diphosphate.

sites, followed by (ii) spontaneous conversion of the enzyme to a form, $E_2 \cdot (K)$, containing occluded K^+.

5. Mg^{2+} or oligomycin slows the rate of conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$ but does not significantly affect the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$.

6. In the light of these and previous findings, we propose a model for the sodium pump in which conformational changes alternate with trans-phosphorylations, and the inward and outward fluxes of both Na^+ and K^+ each involve the transfer of a phosphoryl group as well as a change in conformation between E_1 and E_2 forms of the enzyme or phosphoenzyme.

Introduction

The effects of Na^+ and K^+ on nucleotide binding to $(Na^+ + K^+)$-ATPase [1,2], and on the pattern of tryptic digestion of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) [3], show that the enzyme can exist in either of two forms depending on whether Na^+ or K^+ is the predominant cation. The Na-form has a high affinity for nucleotides, and corresponds to E_1 in the Albers-Post scheme (see ref. 4, and Fig. 1 of the preceding paper [5]); the K-form has a low affinity for nucleotides and corresponds to E_2 in that scheme. The interconversion occurs in the absence of phosphorylation. This paper reports experiments with $(Na^+ + K^+)$-ATPase prepared from pig kidney outer medulla, in which we have followed the interconversion of the two forms by taking advantage of the changes in fluorescence observed when FTP or FDP bind to the enzyme. When K^+ is added in sufficient quantity to a suspension of enzyme with bound formycin nucleotide, the rate of fall of fluorescence gives a measure of the rate of conversion of $E_1 \cdot Na \cdot FDP$, say, to $E_2 \cdot (K)$ * plus FDP. When sufficient Na^+ is added to a suspension of the enzyme in a medium containing K^+ and, say, FDP, the rate of rise of fluorescence gives a measure of the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na \cdot FDP$. With a stopped-flow fluorimeter we have therefore been able to estimate the rates of conversion in each direction under a variety of conditions.

A preliminary account of some of the experiments reported here has already been published [6].

Materials and Methods

These are described in the preceding paper [5] or in ref. 6.

Results

Displacement of nucleotide by K^+ and other ions

When KCl is added slowly to a suspension of $(Na^+ + K^+)$-ATPase in a medium

* Expressions like $E_1 \cdot Na$ and $E_2 \cdot (K)$ are not intended to give any indication of stoichiometry. $E_1 \cdot Na$ merely shows that the enzyme is in the form with high affinity for nucleotides and that sodium is bound to it. The brackets in $E_2 \cdot (K)$ indicate that K^+ bound to the E_2 form of the enzyme are believed not to exchange with K^+ in the medium. The justification for this belief is given in Discussion.

containing FDP and a low concentration of Na^+, there is a progressive fall in fluorescence as the equilibrium

$$K^+ + E_1 \cdot Na \cdot FDP \rightleftharpoons E_2 \cdot (K) + FDP + Na^+$$

is shifted to the right [6]. A similar effect can be observed with FTP, provided that its hydrolysis is prevented by the exclusion of Mg^{2+}. In experiments using a medium containing 4 μM FTP or FDP, 133 μM NaCl, 1 mM EDTA and 80 mM Tris/Tris · HCl (pH 7.7 at 20°C) we found that the concentrations of different cations required to displace half of the nucleotide were, respectively: Tl^+ 0.111 mM; K^+ 0.134 ± 0.003 mM (n = 6); Rb^+ 0.142 mM; NH_4^+ 0.71 mM; Cs^+ 1.3 mM. Li^+ at low concentrations had little effect. The order of effectiveness of the cations is similar to that reported by Hegyvary and Post [1] using more direct methods for measuring nucleotide binding, and is therefore compatible with our working assumption that the changes in fluorescence caused by altering the balance between Na^+ and K^+ simply reflect interconversions between forms of the enzyme with different nucleotide affinities. With 80 mM Na^+ present, approx. 12 mM K^+ was needed to displace 50% of the nucleotide. At higher nucleotide concentrations we should expect the equilibrium to be displaced towards the E_1 form of the enzyme, but this was not tested because the fluorescence changes would have been difficult to detect, and binding of nucleotide to the low-affinity K-form of the enzyme would have made interpretation difficult.

Conversion of K-form to Na-form

When Na^+ in high concentrations is rapidly added to enzyme suspended in a medium containg K^+ in low concentration, the enzyme changes from the K-form $E_2 \cdot (K)$ to the Na-form $E_1 \cdot Na$. If FTP or FDP is present at a concentration well below the K_D for the K-form but above the K_D for the Na-form, the conversion is accompanied by the net binding of nucleotide, and this can be

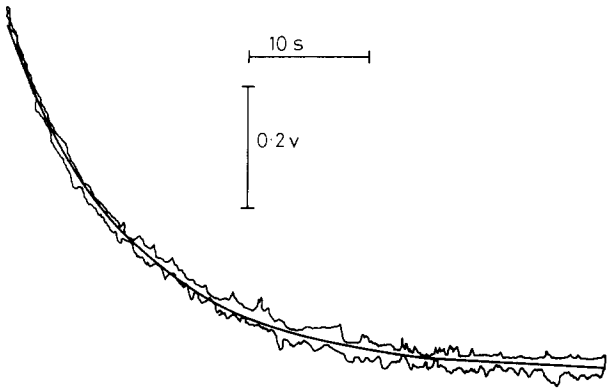

Fig. 1. The curve shows the slow rise in fluorescence which occurred as enzyme in the K-form was converted to the Na-form and bound FTP. Syringe I contained, in a volume of 3 ml: 1.38 mg enzyme protein (specific activity 15 μmol · mg^{-1} · min^{-1}), 0.25 mM Tl_2SO_4, 100 mM Tris/Tris · HCl (pH 7.7), 1 mM EDTA (Tris salt). Syringe II contained 3 ml of a solution containing: 4 μM FTP, 80 mM NaCl, 20 mM Tris/Tris · HCl (pH 7.7), 1 mM EDTA (Tris salt). The temperature was 21°C. The time constant of the amplifier was 50 ms. The figure reproduces the outline of the oscilloscope trace, and the smooth curve represents the equation: $y = y_0 \exp(-0.096\ t)$.

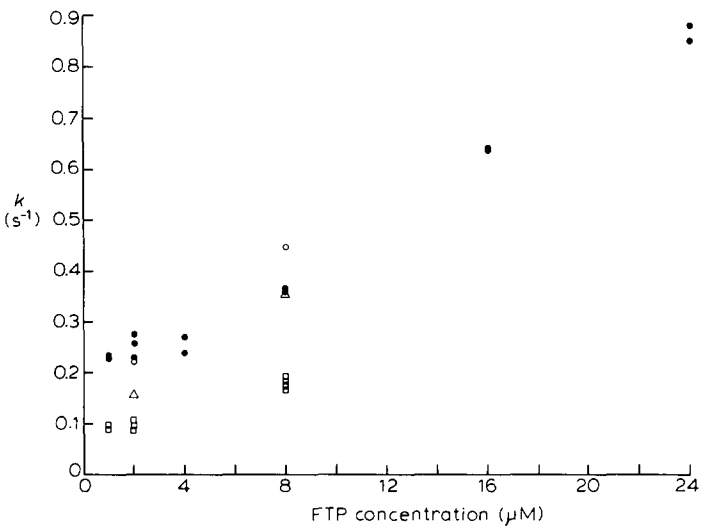

Fig. 2. The effect of FTP concentration on the rate of conversion of $E_2 \cdot (X)$ to $E_1 \cdot Na$, where X is K^+, Tl^+ or Cs^+. The solid symbols are from a single experiment using K^+; the open symbols are from a second experiment using K^+ (○), Tl^+ (□) or Cs^+ (△). In the first experiment, syringe I contained, in a volume of 3 ml: 810 μg enzyme protein (specific activity 14 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), 0.84 mM KCl, 1 mM EDTA (Tris salt), 87 mM Tris/Tris · HCl (pH 7.7), 4 mM histidine, Na^+ nominally zero: syringe II contained 3 ml of a solution containing 100 mM NaCl, 4 mM Tris/Tris · HCl (pH 7.7), and an appropriate concentration of FTP (triethylamine salt). In the second experiment, syringe I contained, in a volume of 3 ml: 1.38 mg enzyme protein (specific activity 15 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), 1 mM KCl or 0.5 or 2 mM TlCl or 20 mM CsCl, 1 mM EDTA (Tris salt), 5 mM histidine, 100 mM Tris/Tris · HCl (pH 7.7), 400 μM NaCl; syringe II contained 3 ml of a solution containing: 80 mM NaCl, 1 mM EDTA, 20 mM Tris/Tris · HCl (pH 7.7) and an appropriate concentration of FTP (triethylamine salt). The temperature was 21°C in both experiments.

observed as an increase of fluorescence. An experiment of this kind, but using Tl^+ instead of K^+, is illutsrated in Fig. 1. (In this experiment, EDTA was present to chelate Mg^{2+}, and the FTP was added with the Na^+ in order to avoid any hydrolysis that might have occurred during prolonged prior exposure to the enzyme.) The increase in fluorescence was remarkably slow and could be fitted well by a single exponential, giving an observed rate constant of approx. 0.1 s^{-1}.

Effect of nucleotide concentration. Fig. 2 summarises the results of two experiments in which the effects of varying the concentration of nucleotide were investigated. In the first experiment (solid symbols), Na^+ and FTP were added to the enzyme in the K-form. The FTP concentration was varied to give final values of from 1 to 24 μM. Although there was a good deal of scatter, it is clear that the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$ was increased as the FTP concentration was raised over most of the range examined. This suggests that the combination of nucleotide with $E_2 \cdot (K)$ at a low-affinity site accelerates its conversion to $E_1 \cdot Na$. In the second experiment (open symbols), Na^+ and FTP were added to enzyme suspended in media containing K^+, Tl^+ or Cs^+. As in the first experiment, raising the nucleotide concentration accelerated the conversion; in addition, at any given nucleotide concentration, the rate of conversion was slower when Tl^+ was substituted for K^+.

Lack of effect of Mg^{2+} and of oligomycin. In the experiments of Figs. 1 and 2, the concentration of Mg^{2+} must have been extremely low, as the medium

contained 1 mM EDTA and nominally no magnesium. Using 4 μM FDP and 1 mM K^+, we did an experiment (similar to the second experiment described in the legend to Fig. 2) to test whether Mg^{2+} affected the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$. The rate constants for the increase in fluorescence were: 0.295 s^{-1} (S.D. = 0.011, n = 3) with 1 mM EDTA and no Mg, 0.296 s^{-1} (S.D. = 0.017, n = 4) with 1 mM EDTA and 2 mM Mg, and 0.276 s^{-1} (S.D. = 0.010, n = 4) with no EDTA and 1 mM Mg.

We also tested the effect of pretreating the enzyme with oligomycin (240 μg enzyme protein incubated at 37°C for 1 h in 1 ml of a solution containing: 0.5 mM NaCl, 100 mM Tris/Tris · HCl (pH 7.7 at 20°C), 1 mM EDTA, 0.61% (v/v) ethanol, and 92 μg oligomycin). The rate of increase in fluorescence was not significantly changed, even though ATPase activity had been inhibited by 77%.

Conversion of Na-form to K-form

When K^+ is added to enzyme suspended in a medium containing a low concentration of Na^+ and a low concentration of FDP, there is a net release of bound FDP and hence a decrease in fluorescence. Fig. 3 shows the results of

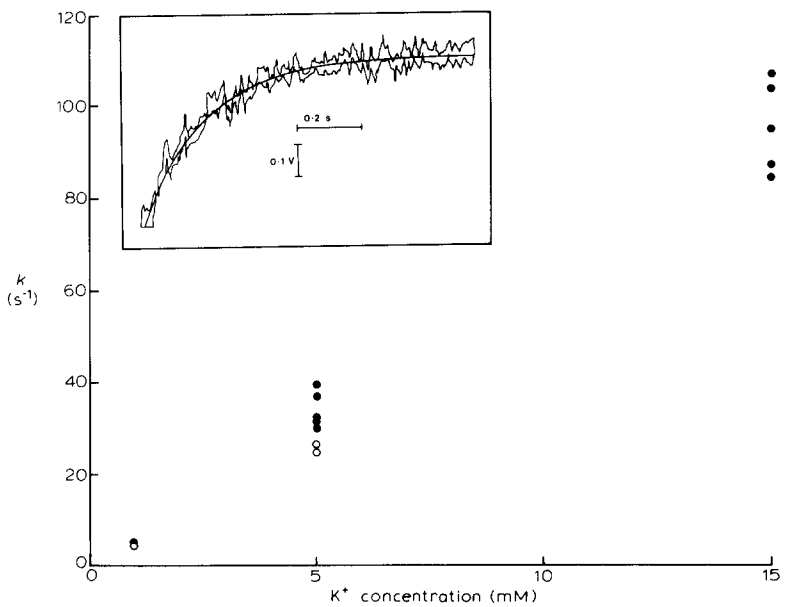

Fig. 3. The effect of K^+ concentration on the rate of conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$. The different symbols denote different experiments. (At 1 mM K^+, the solid symbol represents the mean of five points all lying within it, the open symbol the mean of two points lying within it). In both experiments, syringe I contained, in a volume of 2.5 ml: 945 μg enzyme protein (specific activity 19 μmol · mg^{-1} · min^{-1}), 4 μM FDP (triethylamine salt); 240 μM NaCl, 1 mM EDTA (Tris salt), 88 mM Tris/Tris · HCl (pH 7.7), 3 mM histidine; syringe II contained 2.5 ml of a solution containing: KCl at twice the concentration shown on the abscissa, 4 μM FDP (triethylamine salt), 1 mM EDTA (Tris salt), 100 mM Tris/Tris · HCl (pH 7.7). The temperature was 24°C in the experiment represented by solid symbols and 21°C in the experiment represented by open symbols. Inset: Outline of one of the oscilloscope traces obtained with 1 mM K^+. The smooth curve represents the equation: $y/y_\infty = 1 - \exp(-5.29\ t)$.

TABLE I

THE EFFECT OF Mg^{2+} ON THE RATE OF CONVERSION OF $E_1 \cdot Na$ TO $E_2 \cdot (K)$

The contents of both syringes were similar to those described in the legend to Fig. 3, except that in Experiment 150976 the FDP concentration was 6 μM and the Na^+ concentration was 500 μM. When Mg^{2+} was present, in Experiment 240676 its concentration was 1 mM in both syringes and EDTA was omitted; in Experiment 150976 its concentration was 2 mM but both syringes also contained 1 mM EDTA.

Experiment	Temperature (°C)	EDTA (mM)	Mg^{2+} total (mM)	K^+ (mM)	Rate constant for decrease in fluorescence (s^{-1})
240676	25	1	0	1	5.43 ± 0.11 (n = 3)
		0	1	1	1.29 ± 0.12 (n = 5)
150976	16	1	0	1	1.78 ± 0.03 (n = 5)
		1	2	1	0.82 ± 0.03 (n = 3)

two experiments in which the rate of fall in fluorescence was measured at different K^+ concentrations. The striking feature of the results is that, although under the conditions of these experiments 1 mM K^+ displaces nucleotide almost completely, as indicated by the nearly maximal change in fluorescence observed in steady-state experiments, the rate of the fluorescence change increased linearly with K^+ concentration at least up to 15 mM K^+. At 30 mM (not shown), the rate was still faster but the trace became too noisy to measure.

Effect of Mg^{2+}. Although, as shown above, Mg^{2+} had no effect on the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$, they appeared to slow the conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$. Table I summarises the results of two experiments in which the rate of displacement of FDP by the addition of K^+ was measured both in the presence and in the absence of Mg^{2+}. In both experiments there was a significant effect, though its magnitude differed. We do not know whether this difference was the result of the difference in temperature, the use of different enzyme preparations, or even, conceivably, the presence of mgEDTA in the second experiment [7].

Effect of oligomycin. Pretreatment of the enzyme with oligomycin, as described on p. 256, also slowed the change in fluorescence observed when FDP was displaced by the addition of K^+. With the oligomycin-treated enzyme, the initial fall in fluorescence was at about half the normal rate, but the results are difficult to interpret because the fall could not be fitted by a single exponential (not shown).

Discussion

Conversion of K-form to Na-form

The most striking finding reported here is the remarkable slowness of the rise in fluorescence when the addition of Na^+ to enzyme in the K-form leads to the binding of formycin nucleotides. The rate-limiting step could be the binding of Na^+ to or the dissociation of K^+ from their binding sites, the conformational change, or the subsequent binding of nucleotide. The last possibility may be disregarded since the rate constant for net binding of FTP (at 4 μM) to the Na-form of the enzyme is much too fast (84.4 s^{-1}, see preceding paper [5]). In

general one would not expect the binding and release of Na^+ and K^+ to be slow, and there is evidence from phosphorylation experiments [8,9] and from the observed rates of K^+-dependent displacement of FDP (Fig. 3) that these ions interact rapidly with their sites in the E_1 form of the enzyme. A very slow binding of Na^+ to, or dissociation of K^+ from, binding sites in the E_2 form of the enzyme cannot be ruled out, but it seems more likely that the rate-limiting step is a slow conformational change.

Many years ago, Skou [10] pointed out that the hypothesis that the normal pump cycle consists of a Na^+-dependent phosphorylation followed by a K^+-dependent hydrolysis had to be reconciled with the observation that under some conditions, including, as it later turned out, very low ATP concentrations, K^+ reduced the steady-state level of phosphoenzyme without accelerating the splitting of ATP. If the phosphoenzyme were an intermediate in the pump cycle, this implied that K^+, as well as accelerating the hydrolytic step, slowed some other step. The experiments of Post et al. [11], of Mårdh and Zetterqvist [12] (see p. 26 of ref. 4), and of Mårdh [13,14] provided evidence that the slow step followed the hydrolytic step and, because its rate depended on which congener of K^+ had catalysed the hydrolysis, Post et al. [11] postulated that the slow step involved a conformational change that released K^+ from a form of the enzyme in which they were occluded. Since this slow step was not apparent at high ATP concentration, they also postulated that it was accelerated by ATP acting at a low-affinity site. Our experiments provide strong support for this hypothesis by showing, first, that the conversion of the enzyme from a K-form to an Na-form may indeed be very slow, and, secondly, that it is accelerated by FTP acting with a low affinity. It is obviously economical to assume that the K-form studied in our experiments is identical with the occluded K-form postulated by Post et al. [11], and that it can be generated either by the K^+-catalysed hydrolysis of phosphoenzyme or directly from the Na-form by suitable adjustment of the $Na^+ : K^+$ ratio.

Conversion of Na-form to K-form

Here the rate-limiting step could be the binding of K^+ to or the dissociation of Na^+ from their binding sites, the conformational change, or the subsequent release of FDP. We cannot measure the rate of release of FDP from $E_2 \cdot (K) \cdot$ FDP, but it is likely to be much faster than the release of FDP from $E_1 \cdot Na \cdot$ FDP ($112\ s^{-1}$, see preceding paper [5]) because the binding constant of the complex $E_2 \cdot (K) \cdot$ FDP is at least ten times smaller than the binding constant of the complex $E_1 \cdot Na \cdot$ FDP [5]. Since the observed rate constant for the fall in fluorescence at, say, 5 mM K^+, was less than $40\ s^{-1}$ (see Fig. 3), the release of FDP is probably not rate limiting. The possibility that the binding of K^+ is rate limiting cannot be excluded, and would provide a simple explanation of the linear relation between K^+ concentration and the rate of the fluorescence change. As we show below, however, this relation can also be explained simply if the K^+ rapidly equilibrates with low-affinity binding sites and the conformational change is rate limiting. Again, on general grounds, this seems the more likely explanation.

Fig. 4 shows possible pathways for the conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$ if we do not assume that the K^+ in $E_2 \cdot (K)$ is occluded. For the sake of simplicity,

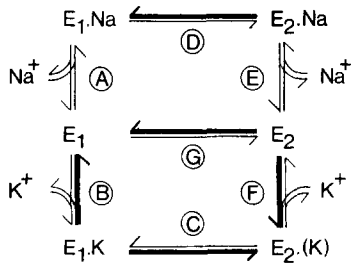

Fig. 4. Possible pathways for the interconversion of $E_1 \cdot Na$ and $E_2 \cdot (K)$, without phosphorylation, if we do not assume that the K^+ in $E_2 \cdot (K)$ are occluded. (For simplicity, multiple ion binding has been ignored). The heavy horizontal arrows indicate the poise of the equilibria when nucleotides are absent, or present at low concentration. Because in any cycle the product of the velocity constants in a clockwise direction must equal the product of the velocity constants in an anti-clockwise direction, the fact that K^+ stabilizes the E_2 form implies that E_2 has a much higher affinity for K^+ than E_1. This is indicated by the heavy vertical arrows. ATP, without phosphorylating, displaces the equilibrium towards $E_1 \cdot Na$; theoretically, it could do this by displacing the horizontal equilibria to the left or the vertical equilibria upwards.

the binding of Na^+ and of K^+ to the sites at which they influence the $E_1 \rightleftharpoons E_2$ equilibrium are regarded as mutally exclusive; this is not improbable, but we do not know whether it is the real situation. Nucleotide binding is not shown in the figure but, at the low nucleotide concentrations with which we are immediately concerned, the E_1 forms would be nearly saturated, and the E_2 forms would scarcely bind any. Essentially, the transition from E_1 to E_2 may occur after the displacement of Na^+ by K^+ (pathway ABC) or before that displacement (pathways DEF or AGF). It is important to note that the virtually complete displacement of the equilibrium towards $E_2 \cdot (K)$ by low concentrations of K^+ does not imply that the sequence is one in which the K^+ reacts with a site of high affinity. For the apparent K_m for K^+ will be a factor not only of the equilibrium constant of the step at which K^+ binds, but of all the equilibrium constants along the reaction pathway. More specifically, if we consider the isolated sequence:

$$E_1 + K^+ \rightleftharpoons E_1 \cdot K \rightleftharpoons E_2 \cdot (K)$$

it is easy to show that the concentration of K^+ at which half of the enzyme is in the form $E_2 \cdot (K)$ is given by the expression $K_1/(K_2 - 1)$, where $K_1 = [E_1][K^+]/[E_1 \cdot K]$ and $K_2 = [E_2 \cdot (K)]/[E_1 \cdot K]$ *. Whatever the magnitude of K_1, the magnitude of this expression can be very low provided that K_2 is large enough, i.e. provided that the equilibrium $E_1 \cdot K \rightleftharpoons E_2 \cdot (K)$ is poised far enough to the right.

Because the pathway ABC contains this sequence, it can account both for the high apparent affinity for K^+ in steady-state experiments (see p. 254), which give information about the equilibrium between $E_1 \cdot Na$ and $E_2 \cdot (K)$ and the very low apparent affinity for K^+ in experiments like those of Fig. 3, which measure the effect of K^+ on the rate of the transition from $E_1 \cdot Na$ to $E_2 \cdot (K)$.

* For the derivation of this expression, and for the calculations that follow, we assume that, in the relevant range of K^+ concentrations, the binding of one K^+ at one site is critical for the poise of the equilibrium between the two conformations. The linear relation between the rate of the fluorescence change following K^+ addition and the final K^+ concentration suggests that this assumption is correct (see page 257).

For the observed rate of the transition will depend not only on the rate constant for the conformational change $E_1 \cdot K \rightarrow E_2 \cdot (K)$, but also on the fraction of E_1 that is occupied by K^+. If the conformational change is improbable unless K^+ is bound, we may write

$$k_{obs} = k_{+2}[K^+]/(K_1 + [K^+]) + k_{-2},$$

where k_{obs} is the observed rate constant, k_{+2} and k_{-2} are the forward and backward rate constants for the conformational change $E_1 \cdot K \rightleftharpoons E_2 \cdot (K)$ and K_1 is the effective dissociation constant for the reaction $E_1 \cdot K \rightleftharpoons E_1 + K^+$ at the prevailing Na^+ concentration. Provided that $[K^+]$ is small compared with K_1, this will give a linear relation between the observed rate and the K^+ concentration with an intercept on the y-axis equal to k_{-2}.

To determine k_{+2}, it would be necessary to measure the rate of the fluorescence change at saturating K^+ concentrations. This is not possible, but the value is clearly greater than the fastest measurable rate, which was about 100 s^{-1}. The value of k_{-2} must be small because the intercept in Fig. 3 is near the origin, but we can get a much better estimate from Fig. 2 if we assume that the observed rate constant for the change $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ is determined by the rate of the step $E_2 \cdot (K) \rightarrow E_1 \cdot K$ rather than the rate of exchange of cations. The results shown in Fig. 2 suggest that at low nucleotide concentrations k_{-2} must be about 0.2 s^{-1}, i.e. at least 500 times smaller than k_{+2}. The ratio of the forward rate constant to the backward rate constant is a measure of the extent to which the equilibrium between $E_1 \cdot K$ and $E_2 \cdot (K)$ is poised in favour of $E_2 \cdot (K)$.

The assumption that the transition from $E_1 \cdot Na$ to $E_2 \cdot (K)$ occurs by the pathway ABC is, therefore, compatible with all the experimental results. The alternative pathways (DEF and AGF) in Fig. 4 are unlikely, as can be seen from the following argument. Studies of nucleotide binding [1,2] and of trypsin digestion [3] both suggest that in the absence of K^+ the enzyme is in the E_1 form, whether Na^+ is present or not. This implies that the equilibria $E_1 \rightleftharpoons E_2$ and $E_1 \cdot Na \rightleftharpoons E_2 \cdot Na$ are both poised well to the left. However, we know that, at least at low nucleotide concentrations, the rate of conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$ is very much faster than the rate of conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$. If the interconversion of $E_1 \cdot Na$ and $E_2 \cdot (K)$ were along either of the pathways DEF or AGF it would follow that the slow rate-limiting step in the conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$ was not a step involving a change in conformation but a step involving the dissociation or association of a small ion. This seems improbable.

Because $(Na^+ + K^+)$-ATPase activity does not require extracellular Na^+ [15], we presume that it is intracellular Na^+ that promote phosphorylation of the E_1 form of the enzyme by ATP. Provided that K^+ acts simply by replacing Na^+ at the internal Na^+-loading sites, which is the most straightforward hypothesis and is also suggested by the low affinity for K^+ observed in the kinetic experiments (Fig. 3), only K^+ at the inward-facing surface of the membrane will be effective at converting $E_1 \cdot Na$ to $E_1 \cdot K$. If the conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$ follows the sequence ABC of Fig. 4, we may therefore predict that it will not occur, or will be much slower, if K^+ is present only at the outward-facing surface. (This is, of course, in contrast to the formation of $E_2 \cdot (K)$ by the hydrolysis of phosphoenzyme, which requires extracellular K^+). This prediction is, theoretically,

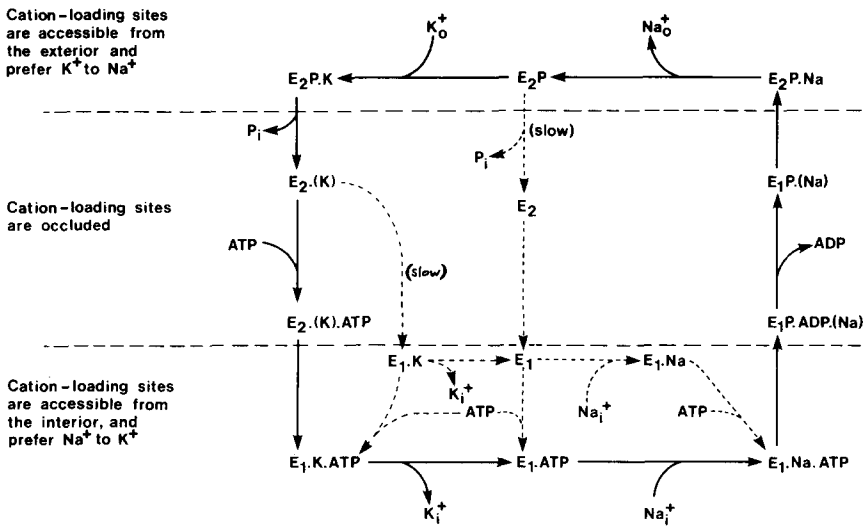

Fig. 5. Scheme showing the proposed relations between ion movements, phosphoryl transfers and conformational changes. The solid arrows show the events supposed to take place in physiological conditions, when Na^+-K^+ exchange occurs in the presence of ATP at a concentration high enough to allow binding at the low-affinity binding site of the enzyme. When the pump is made to run backwards, each step is reversed and the order is reversed. Na^+-Na^+ exchange is explained by the alternate forward and backward running of the right-hand part of the scheme, and K^+-K^+ exchange by the alternate forward and backward running of the left-hand part of the scheme. Uncoupled Na^+ efflux is brought about by an outward movement through the right-hand part of the scheme, the cycle being completed via the central dotted pathway. All movements of ions across the membrane require a phosphoryl transfer, as well as a change in conformation between the E_1 and E_2 forms of either enzyme or phosphoenzyme. Combination with ATP and release of ADP and P_i take place at the intracellular surface of the membrane. For simplicity, multiple binding of the transported ions has been ignored. Note that: (i) There is no evidence about whether the site that binds ATP with a low affinity, when the enzyme is in the $E_2 \cdot (K)$ form, is itself converted to the high-affinity ATP binding site by the conformational change. (ii) Although the sequence of chemical events described by the scheme is the same as in recent versions of the Albers-Post scheme (see in Fig. 2 of ref. 38 or Fig. 1 of preceding paper [5]), the relations between ion movements and conformational changes are quite different from those proposed by Post and his colleagues (see Fig. 17 of ref. 38).

testable, though we have not yet succeeded in incorporating sufficient enzyme into sufficiently tight artificial lipid vesicles for the test to be made.

The hypothesis that in the absence of phosphorylation $E_2 \cdot (K)$ is formed from intracellular K^+ via $E_1 \cdot K$ and does not exchange readily with extracellular K^+, also fits well with the properties of the K^+-K^+ exchange catalysed by the sodium pump in red cells. This is known to require intracellular orthophosphate [16] and either ATP [17] or its $\beta\gamma$-imido or methylene analogue [17]. Presumably the orthophosphate is needed to phosphorylate the $E_2 \cdot (K)$ so that the K^+ may have access to the external solution; and the ATP, or its analogue, is needed to accelerate the conversion of $E_2 \cdot (K)$ to $E_1 \cdot K$ (see Fig. 5). The known asymmetry of the K^+ requirements on the two sides of the membrane [16,19], and the existence of a K^+-stimulated exchange of ^{18}O between water and orthophosphate [20] are also compatible with the hypothesis.

The relation of the conformational changes to events in the normal pump cycle

Next, we must consider the relation of the changes that we have been study-

ing, which occur in the absence of phosphorylation, to those that are supposed to occur during the normal working of the ($Na^+ + K^+$)-ATPase as an ATP-driven ion pump (see Fig. 5). Under physiological conditions, the concentrations of the various ligands are such that if phosphorylation did not occur the enzyme would be expected to be predominantly in the E_1 form, binding both Na^+ and ATP [1,3]. Phosphorylation by ATP alters the equilibrium so that the E_1P formed changes spontaneously to E_2P [21,22]. The properties of the Na^+-Na^+ exchange, and of the uncoupled Na^+ efflux catalysed by the pump under certain unphysiological conditions [4,23—36], make it reasonable to assume that this conformational change is associated with the outward movement of Na^+. Hydrolysis of E_2P, catalysed by extracellular K^+, is then thought to form $E_2 \cdot (K)$ which reverts spontaneously to $E_1 \cdot K$ at a rate which is accelerated by the binding of ATP at a low-affinity site (ref. 11; and this paper). As we have seen, the properties of K^+-K^+ exchange make it reasonable to assume that the conformational change $E_2 \cdot (K) \cdot ATP \rightarrow E_1 \cdot K \cdot ATP$ precedes the release of K^+ at the inner surface of the membrane. Finally, the cycle is supposed to be completed by the displacement of K^+ by intracellular Na^+. Thus the conversion of $E_2 \cdot (K) \cdot ATP$ to $E_1 \cdot Na \cdot ATP$ via $E_1 \cdot K \cdot ATP$ appears to be an essential part of the normal Na^+-K^+ exchange cycle. The reverse transformation, $E_1 \cdot Na \cdot ATP \rightarrow E_1 \cdot K \cdot ATP \rightarrow E_2 \cdot (K) \cdot ATP$, would not be expected to form part of the Na^+-K^+ exchange cycle, but it is, we believe, involved in the K^+-K^+ exchange which, in red cells at least, accompanies the Na^+-K^+ exchange to a small extent under physiological conditions [37]. With or without the bound nucleotide it also, presumably, forms part of the cycle when ATP is synthesized at the expense of energy from ion gradients by an artificial reversal of the pump mechanism.

It is worth noting that, though there are reasons for believing that K^+ movements involve conformational changes between $E_1 \cdot K \cdot ATP$ and $E_2 \cdot (K) \cdot$

TABLE II

CONFORMATIONAL CHANGES AND PHOSPHORYL TRANSFERS ASSOCIATED WITH CATION FLUXES CATALYSED BY ($Na^+ + K^+$)-ATPase

Flux	Na^+ efflux	Na^+ influx	K^+ influx	K^+ efflux
Modes in which flux occurs	Na^+-K^+ exchange Na^+-Na^+ exchange Uncoupled Na^+ efflux	Reversed mode Na^+-Na^+ exchange	Na^+-K^+ exchange K^+-K^+ exchange	Reversed mode K^+-K^+ exchange
Requirements	ATP, Mg^{2+}, Na^+; (K_o^+ for Na^+-K^+ exchange) (Na_o^+ and ADP for Na^+-Na^+ exchange)	ADP, Mg^{2+}, Na_o^+; (P_i and K_i^+ for reversed mode) (ATP and Na_i^+ for Na^+-Na^+ exchange)	ATP *, Mg^{2+}, K_o^+; (Na_i^+ for Na^+-K^+ exchange) (P_i and K_i^+ for K^+-K^+ exchange)	P_i, Mg^{2+}, K_i^+; (ADP and Na_o^+ for reversed mode) (ATP * and K_o^+ for K^+-K^+ exchange)
Conformational change	$E_1P \cdot (Na) \rightarrow E_2P \cdot Na$	$E_2P \cdot Na \rightarrow E_1P \cdot (Na)$	$E_2 \cdot (K) \cdot ATP \rightarrow E_1 \cdot K \cdot ATP$	$E_1 \cdot K \cdot ATP \rightarrow E_2 \cdot (K) \cdot ATP$
Phosphoryl transfer	From ATP to $E_1 \cdot Na$	From $E_1P \cdot (Na)$ to ADP	From $E_2P \cdot K$ to H_2O	From P_i to $E_2 \cdot (K)$
References	4,23,25—36	4,23,25,28—30 32—35, 51—55	4,16—19,28,29	4,16—19,28,29 35—38, 51—56

* AMP-P(NH)P and AMP-P(CH_2)P can substitute for ATP in supporting K^+-K^+ exchange [18].

ATP and that Na^+ movements involve conformational changes between $E_1P \cdot Na$ and $E_2P \cdot Na$ (see Table II), it is doubtful whether conformational changes without phosphoryl transfer can produce even passive movements of K^+ or Na^+ through the $(Na^+ + K^+)$-ATPase system. Since orthophosphate is required for K^+-K^+ exchange [16] but not for Na^+-K^+ exchange, it must be necessary for K^+ efflux; we suspect that its role is to phosphorylate $E_2 \cdot (K)$. Since ADP is required for Na^+-Na^+ exchange [24,25] but not for Na^+-K^+ exchange, it must be necessary for Na^+ influx; we suspect that its role is to accept a phosphoryl group from $E_1P \cdot Na$. The need for both phosphoryl transfers, can be explained most simply by supposing that only dephosphoenzyme in the E_1 form can pick up or discharge the transported ions at the inner surface of the membrane, and that only phosphoenzyme in the E_2 form can pick up or discharge the transported ions at the outer surface. In this scheme, $E_1P \cdot Na$ therefore represents an occluded form of Na^+, corresponding to $E_2 \cdot (K)$, the occluded form of K^+. For this reason, it is written $E_1P \cdot (Na)$ in Fig. 5 and Table II.

Attractive though the scheme of Fig. 5 is, there is another hypothesis which is worth mentioning. ATP or its non-phosphorylating analogues make K^+-K^+ exchange possible by accelerating the conversion of $E_2 \cdot (K)$ to $E_1 \cdot K$ which, as we have seen, is extremely slow at low nucleotide concentrations. It is conceivable that Na^+-Na^+ exchange depends in a parallel fashion on the acceleration of the conversion of $E_2P \cdot Na$ to $E_1P \cdot Na$, and that ADP is necessary as accelerator because the binding of ATP is prevented by the phosphoryl group already on the enzyme. In other words, the role of ADP may not be to act as a phosphoryl acceptor from $E_1P \cdot Na$ but to combine with $E_2P \cdot Na$ and accelerate its conversion to $E_1P \cdot Na$, which is then able to release Na^+ to the cell interior without the intervention of a transphosphorylation step.

Theoretically, it would be possible to decide between these hypotheses, either by detecting inexchangeable Na in $E_1P \cdot (Na)$, or by demonstrating that Na^+-Na^+ exchange could be faster than ATP-ADP exchange. In practice, the technical difficulties are formidable.

The hypotheses that we have been discussing may be criticized for failing to take into account evidence, based on the independence of Na^+ and K^+ affinities on the two sides of the membrane, suggesting that the external K^+-binding sites and the internal Na-binding sites coexist and must be occupied simultaneously before transport can occur (see refs, 38—43). It is therefore worth pointing out that if the pump is a dimer with the two halves appropriately coupled, and with certain other criteria satisfied, it is possible to reconcile independence of the ion affinities with the kind of "consecutive" behaviour illustrated by Fig. 5 (cf. refs. 4, 44—50).

Acknowledgements

We are grateful to Professor H. Gutfreund, Dr. D.R. Trentham and Dr. J.F. Eccleston for advice and help. Different aspects of the work were supported by grants from the British M.R.C. and S.R.C., and by a grant from the American M.D.A. to Dr. Trentham. S.J.D. K. held E.M.B.O. short-term fellowships and was also supported by the Royal Society.

References

1 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
2 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
3 Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 339—415
4 Glynn, I.M. and Karlish, S.J.D. (1975) Ann. Rev. Physiol. 37, 13—55
5 Karlish, S.J.D., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 230—251
6 Karlish, S.J.D., Yates, D.W. and Glynn, I.M. (1976) Nature 236, 251—253
7 Klodos, I. and Skou, J.C. (1975) Biochim. Biophys. Acta 391, 474—485
8 Mårdh, S. and Post, R.L. (1977) J. Biol. Chem. 252, 633—638
9 Tonomura, Y. and Fukushima, Y. (1974) Ann. N.Y. Acad. Sci. 242, 92—105
10 Skou, J.C. (1965) Physiol. Rev. 45, 596—617
11 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540
12 Mårdh, S. and Zetterqvist, O. (1972) Biochim. Biophys. Acta 255, 231—238
13 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448—463
14 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 464—473
15 Glynn, I.M. (1962) J. Physiol. Lond. 160, 18P—19P
16 Glynn, I.M., Lew, V.L. and Lüthi, U. (1970) J. Physiol. Lond. 207, 371—391
17 Glynn, I.M., Hoffman, J.F. and Lew, V.L. (1971) Phil. Trans. R. Soc. B 262, 91—102
18 Simons, T.J.B. (1975) J. Physiol. Lond. 244, 731—739
19 Simons, T.J.B. (1974) J. Physiol. Lond. 237, 123—155
20 Dahms, A.S. and Boyer, P.D. (1973) J. Biol. Chem. 248, 3155—3162
21 Siegel, G.J. and Albers, R.W. (1967) J. Biol. Chem. 242, 4972—4979
22 Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s—326s
23 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. Lond. 192, 189—216
24 Glynn, I.M. and Hoffman, J.F. (1971) J. Physiol. Lond. 218, 239—256
25 Cavieres, J.D. and Glynn, I.M. (1976) J. Physiol. Lond. 263, 214P—215P
26 Karlish, S.J.D. and Glynn, I.M. (1974) Ann. N.Y. Acad. Sci. 242, 461—470
27 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. Lond. 256, 465—496
28 Glynn, I.M. and Karlish, S.J.D. (1975) Ciba Foundation Symp. 31, 205—223
29 Glynn, I.M. and Karlish, S.J.D. (1974) Biochem. Soc. Spec. Publ. 4, 145—158
30 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. Lond. 192, 159—174
31 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. Lond. 192, 175—188
32 Baker, P.F., Blaustein, M.P., Keynes, R.D., Manil, J., Shaw, T.I. and Steinhardt, R.A. (1969) J. Physiol. Lond. 200, 459—496
33 Caldwell, P.C., Hodgkin, A.L., Keynes, R.D. and Shaw, T.I. (1960) J. Physiol. Lond. 152, 561—590
34 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. Lond. 192, 217—235
35 De Weer, P. (1970) J. Gen. Physiol. 56, 583—620
36 Lew, V.L., Hardy, M.A. and Ellory, J.C. (1973) Biochim. Biophys. Acta 323, 251—266
37 Glynn, I.M. (1956) J. Physiol. Lond. 134, 278—310
38 Garay, R.P. and Garrahan, P.J. (1973) J. Physiol. Lond. 231, 297—325
39 Baker, P.F. and Stone, A.J. (1966) Biochim. Biophys. Acta 126, 321—329
40 Hoffman, P.G. and Tosteson, D.C. (1971) J. Gen. Physiol. 58, 438—466
41 Garrahan, P.J. and Garay, R.P. (1976) Curr. Top. Membranes Transport 8, 29—97
42 Skou, J.C. (1973) J. Bioenerg. 4, 1—30
43 Skou, J.C. (1975) Q. Rev. Biophys. 7, 401—434
44 Robinson, J.D. (1974) Ann. N.Y. Acad. Sci. 242, 185—202
45 Stein, W.D., Lieb, W.R., Karlish, S.J.D. and Eilam, Y. (1973) Proc. Natl. Acad. Sci. U.S. 70, 275—278
46 Repke, K.R.H. and Schön, R. (1973) Acta Biol. Med. Ger. 31, K19—K30
47 Siegel, G.J., Goodwin, B.B. and Hurley, M.J. (1974) Ann. N.Y. Acad. Sci. 242, 220—234
48 Repke, K.R.H., Schön, R. and Dittrich, F. (1975) Proc. 9th FEBS Meet. 35, 241—253
49 Repke, K.R.H. (1977) FEBS Symp. 42, 363—373
50 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006—1019
51 Garraham, P.J. and Glynn, I.M. (1967) J. Physiol. Lond. 192, 237—256
52 Glynn, I.M. and Lew, V.L. (1970) J. Physiol. Lond. 207, 393—402
53 Lant, A.F., Priestland, R.N. and Whittam, R. (1970) J. Physiol. Lond. 207, 291—301
54 Lant, A.F. and Whittam, R. (1968) J. Physiol. Lond. 199, 457—484
55 Lew, V.L., Glynn, I.M. and Ellory, J.C. (1970) Nature 225, 865—866
56 Post, R.L., Toda, G., Kume, S. and Taniguchi, K. (1975) J. Supramol. Struct. 3, 479—497