Biochimica et Biophysica Acta, 384 (1975) 250—264

BBA 67435

FUNCTIONALLY DISTINCT CLASSES OF K^+ SITES ON THE ($Na^+ + K^+$)-DEPENDENT ATPase

JOSEPH D. ROBINSON
Department of Pharmacology, State University of New York, Upstate Medical Center Syracuse, N. Y. (U.S.A.)

(Received July 1st, 1974)
(Revised manuscript received October 14th, 1974)

Summary

K^+ interactions with a rat brain ($Na^+ + K^+$)-dependent ATPase and the associated K^+-dependent nitrophenyl phosphatase activity were examined. Classes of sites for K^+ were distinguished, initially, on the basis of affinity estimated by kinetic analysis in terms of $K_{0.5}$ (the concentration for half-maximal activation), and by K^+-accelerated enzyme inactivation by F^-, which permits evaluation of a dissociation constant for K^+, K_D. Moderate-affinity sites ("α sites"), with a K_D near 1 mM, were demonstrable for the phosphatase activity and for the "free" enzyme. High-affinity sites ("β sites"), with a K_D near 0.1 mM, were seen for the overall ATPase activity and under conditions in which enzyme phosphorylation by substrate also occurs. Further differentiation between α and β sites was made in terms of (i) the characteristic changes in affinity with pH, and (ii) the efficacy of Li^+ relative to K^+, Rb^+, Cs^+, and Tl^+ at these two classes of sites. Low-affinity sites ("γ sites") through which K^+ inhibits enzymatic activity were also detectable, with a K_D around 140 mM. These data are incorporated into a model for the reaction sequence to accommodate both transport processes and certain K^+/ATP antagonisms.

Introduction

Although the identification of the ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) with the membrane sodium/potassium pump is convincing [1,2], major difficulties in relating the reaction sequence of the enzyme to the actual transport of the cations remain. This report is concerned with one aspect of this problem, the properties of the K^+ sites of the enzyme and how they may reflect successive stages of the overall reaction sequence. In correspondence with the pump that accepts K^+ at sites on the external membrane surface and releases the transported K^+ into the cytoplasm, the enzyme

in situ is activated by K^+ at sites on the external membrane surface [1,2]. These K^+ sites, which govern the K^+-dependent actions of the enzyme (e.g. enzyme dephosphorylation; phosphatase activity), thus are distinguishable from Na^+ sites found at the inner membrane surface, in correspondence with those of the pump which transport Na^+ out of the cytoplasm, and that govern Na^+-specific actions of the enzyme (e.g. enzyme phosphorylation by substrate).

Previous studies on K^+ interactions with this enzyme, in terms both of activation kinetics [3—5] and of K^+-modified inactivation [6,7], are extended here to document three distinguishable classes of sites for K^+: (i) α sites of moderate affinity through which the phosphatase reaction and enzyme inactivation by Be^{2+} and F^- are stimulated; (ii) β sites of high affinity demonstrable after enzyme phosphorylation as in the overall (Na^+ + K^+)-dependent ATPase reaction; and (iii) inhibitory, low-affinity γ sites. Data on these three classes of sites are interpreted in terms of an enzyme that undergoes conformational changes to facilitate acceptance of K^+ in a high-Na^+ environment (conversion of α to β sites), with transport accomplished through a K^+-specific gateway or channel and ultimate discharge from low-affinity sites (γ sites). This model emphasizes distinct coexisting sites for Na^+ and K^+ [1,4—10] employing modest conformational changes to effect transport. It differs from proposals with cyclical interconversions of sites (Na^+ sites transformed into K^+ sites, and vice versa) that frequently propose major conformational events such as subunit rotation to achieve transport. This model also differs from those requiring ATP binding to promote the release of transported K^+ [11]; nucleotide effects on K^+ binding are interpreted here in terms of enzyme phosphorylation or of alternative pathways.

Methods and Materials

The (Na^+ + K^+)-dependent ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described [3].

(Na^+ + K^+)-dependent ATPase activity was measured in terms of the production of P_i, as previously described [3]. The standard medium contained 30 mM histidine · HCl · Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.1 mg protein per ml). Incubation was for 4—8 min at 37°C; activity was linear with time during these periods. Activity in the absence of Na^+ and K^+ ("Mg^{2+}-ATPase") was measured concurrently; such activity averaged only a few percent of the (Na^+ + K^+)-dependent ATPase activity [3], and was subtracted from the total activity in the presence of Na^+ and K^+ to give the (Na^+ + K^+)-dependent activity. Because of variations in the absolute activity of different enzyme preparations, enzyme velocities are expressed relative to the (Na^+ + K^+)-dependent ATPase activity of a concurrent control incubation in the standard medium, defined as 1.0.

K^+-dependent phosphatase activity was measured in terms of the production of *p*-nitrophenol after incubation with *p*-nitrophenyl phosphate, as previously described [4]. The standard medium contained 30 mM histidine · HCl · Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM nitrophenyl phosphate (as the Tris salt), 10

mM KCl, and the enzyme preparation (0.1 mg protein per ml). Incubation was for 8—15 min at 37°C; activity in the absence of added KCl was measured concurrently; such activity averaged only a few percent of the K^+-dependent phosphatase activity under optimal conditions [4], and was subtracted from the total activity in the presence of KCl to give the K^+-dependent activity. As with the ATPase, velocities are expressed relative to the K^+-dependent phosphatase activity of a concurrent control incubation in the standard medium, defined as 1.0.

The affinity for K^+ was also approached in terms of K^+-accelerated inactivation of the enzyme by F^-. In these experiments the pseudo first-order rate constants for enzyme inactivation were determined as a function of K^+ concentration, by means of initial incubations at 37°C of enzyme (0.5 mg protein per ml), 30 mM histidine · HCl · Tris (pH 7.8), 0.5 mM $MgCl_2$, 1.5 mM LiF, and a range of KCl concentrations (plus other additions as specified). These inactivating incubations were terminated by adding 4 vol. of an ATPase incubation medium so that the final concentrations of reactants were those of the standard medium (above), and the residual activity was then measured during brief incubations (changes in activity during this assay incubation were negligible since the inactivation is essentially irreversible and the dilution of F^- and the added NaCl prevent further inactivation). The kinetic model is identical to that for the inactivation of this enzyme by Be^{2+} [6]: the pseudo first-order rate constants for inactivation, k_{in}, can be treated analogously to initial velocities in enzyme kinetics, and thus plots of k_{in} against KCl concentration in double reciprocal form will permit estimation of the dissociation constant K_D.

ATP, CTP, acetyl phosphate, and nitrophenyl phosphate were purchased from Sigma Chemical Co., and the β, γ-methylene analog of ATP from Miles Laboratories; these were then converted to Tris salts [12]. All solutions were made in water redistilled from an all-glass still. Protein was measured by the biuret method, using bovine serum albumin as a standard.

The data presented are averages of five or more experiments performed in duplicate; enzymatic activity is expressed relative to that of concurrent controls incubated in the standard medium. Values of V and of $K_{0.5}$, the concentration for half-maximal velocity, were obtained by fitting the kinetic data to the Hill equation using the procedure described by Atkins [13].

Results

K^+-stimulated inactivation by F^-

As previously described [14—17], the (Na^+ + K^+)-dependent ATPase is inactivated by F^- in the presence of Mg^{2+}, and this inactivation is markedly stimulated by K^+. Although incubation of the enzyme with 0.5 mM Mg^{2+} and 1.5 mM F^- resulted in only a slight decrease in activity after 8 min (less than 10%), as measured by subsequent incubation in the standard medium, the presence of K^+ with Mg^{2+} and F^- sharply increased the rate of inactivation (Fig. 1). Inactivation followed a first-order time course, and as in analogous experiments with K^+-stimulated inactivation by Be^{2+} [6] plots of the pseudo first-order rate constants for inactivation against KCl concentration, in double-reciprocal form, permit estimation of the dissociation constant for K^+ from the

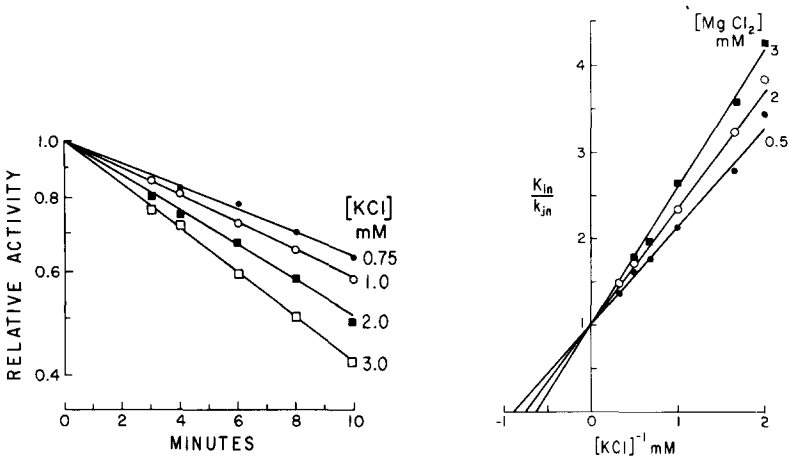

Fig. 1. Effect of KCl on inactivation of the $(Na^+ + K^+)$-dependent ATPase by F^-. The enzyme preparation was first incubated with 30 mM histidine · HCl · Tris (pH 7.8), 1.5 mM LiF, and 0.5 mM $MgCl_2$ in the absence or presence of KCl: 0.75 mM (●), 1.0 mM (○), 2.0 mM (■), or 3 mM (□). This inactivating incubation was terminated at the times indicated by adding 4 vol. of media to bring the final concentration of reactants to their standard concentrations: 30 mM histidine · HCl · Tris (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl. The remaining activity was measured, in terms of production of P_i, after further incubation for 6—8 min in this medium. Concurrent assay incubations without added NaCl and KCl were also performed to monitor any changes in Na^+- and K^+-independent ATPase activity. The data are presented in terms of the $(Na^+ + K^+)$-dependent ATPase activity remaining after inactivating incubations of 3—10 min, relative to corresponding incubations in the absence of KCl.

Fig. 2. Effect of $MgCl_2$ on the apparent K_D for K^+. Experiments were performed as in Fig. 1. Inactivating incubations in the absence or presence of the KCl concentrations indicated were performed with 0.5 mM (●), 2.0 mM (○), and 3.0 (■) $MgCl_2$, and the remaining $(Na^+ + K^+)$-dependent ATPase activity measured by assay incubations in the standard medium. Pseudo first-order rate constants for inactivation, k_{in}, were obtained (as Fig. 1), and these are here plotted against the KCl concentration in double reciprocal form. The rate constants for inactivation are normalized by dividing by K_{in}, the maximal rate constant at infinite KCl.

sites controlling inactivation, K_D (Fig. 2). In this manner an apparent K_D for K^+ of 1.1 mM is obtained (Table I), in accord (Table II) with that from studies on K^+-stimulated inactivation by Be^{2+} and near the $K_{0.5}$ for K^+ activation of the K^+-dependent phosphatase activity of this enzyme [4—7]. Further correspondence of the K^+ sites governing inactivation with those of the phosphatase activity is indicated by similar values for the K^+ analogs Tl^+ and NH_4^+ (Table II).

In addition, both phlorizin and dimethylsulfoxide significantly decreased the K_D for K^+ in terms of inactivation by F^- (Table I), just as with inactivation by Be^{2+} (K_D for K^+ halved by both agents [6]) and with the phosphatase reaction (40% reduction in $K_{0.5}$ by phlorizin [18]; 20% reduction in $K_{0.5}$ by dimethylsulfoxide [19]).

The apparent K_D is a function of the $MgCl_2$ concentration (Fig. 2) and Mg^{2+} apparently competes with K^+ for the K^+ sites (as in all kinetic demonstrations of competitive inhibition actual competition may not exist in reality since the inhibitor could influence affinity through an allosteric site). The K_i for Mg^{2+} is calculated to be 5.1 mM, and thus the true K_D for K^+, in the absence of

TABLE I

APPARENT DISSOCIATION CONSTANTS FOR K^+

The apparent dissociation constant for K^+, K_D, was estimated in terms of K^+-accelerated inactivation by F^-, as indicated in Figs 1 and 2, from plots of the pseudo first-order rate constant for inactivation against KCl concentration. Experiments were performed in the absence of other ligands (Fig. 1), or in the presence of the agents listed. Data are presented ± S.D.

Additions	K_D (mM)
None	1.1 ± 0.2
Phlorizin, 0.03 mM	0.7 ± 0.2*
Dimethylsulfoxide, 10% (v/v)	0.6 ± 0.2*
NaCl, 10 mM	3.7 ± 0.3*
NaCl, 20 mM	5.2 ± 0.4*
Oligomycin, 2 μg/ml	1.0 ± 0.2

* Significantly different from no addition ($P < 0.05$).

Mg^{2+}, would then be 1.0 mM. (With the phosphatase activity Mg^{2+} also acts as a competitor toward K^+ [4].) In addition, Na^+ competes for the K^+ sites (Table I) with an estimated K_i of 5 mM, similar (Table II) to Na^+ inhibition both of inactivation by Be^{2+} [6] and of the phosphatase activity [4,5].

These moderate affinity sites for K^+ seen in the phosphatase reaction or in the inactivation experiments are here termed α sites.

Effects of nucleotides

Adding nucleotides to the inactivating incubation (with K^+, Mg^{2+}, and F^-) increased the K_D for K^+ (Table III); the change was, however, only several-fold.

By contrast, adding nucleotides together with NaCl markedly decreased the K_D for K^+ (Table III). This occurred not only with ATP and CTP but also with acetyl phosphate, all of which are capable of phosphorylating the enzyme in the presence of Mg^{2+} and Na^+ [2,20,21]. On the other hand, the ATP analog

TABLE II

COMPARISON OF KINETIC PARAMETERS

Kinetic parameters from the current experiments are compared with values from previous analogous experiments. Experiments with F^- inactivation were performed in the presence of 0.5 mM $MgCl_2$; other experiments were in the presence of 3 mM $MgCl_2$.

	Kinetic parameter (mM)		
	For inactivation by F^-	For inactivation by Be^{2+}	For phosphatase reaction
K_D or $K_{0.5}$ for K^+	1.1	1.4*	1.9
K_D or $K_{0.5}$ for Tl^+	0.16	0.15*	0.29
K_D or $K_{0.5}$ for NH_4^+	8.6	9.1*	12.6**
K_i for Na^+	5	6*	6***

* Ref. 6.
** Ref. 5.
*** Ref. 4.

TABLE III

EFFECTS OF NUCLEOTIDES ON THE K_D FOR K^+

The experiments were performed and the data are presented as in Table I.

Additions	K_D (mM)	
	Without NaCl	With 10 mM NaCl
None	1.1 ± 0.2	3.7 ± 0.3
MgATP, 0.1 mM	2.2 ± 0.4	0.10 ± 0.03
MgCTP, 0.15 mM	2.3 ± 0.3	0.08 ± 0.02
plus oligomycin, 2 μg/ml	—	0.07 ± 0.02
plus dimethylsulfoxide, 10% (v/v)	—	0.10 ± 0.03
Mg^{2+}/acetyl phosphate	1.4 ± 0.3	0.20 ± 0.06
Mg^{2+}/β,γ-methylene analog of ATP, 0.1 mM	1.4 ± 0.3	4.0 ± 0.5
Mg^{2+}/β,γ-methylene analog of ATP, 0.5 mM	—	4.1 ± 0.5
MgEDTA, 0.1 mM	1.0 ± 0.2	3.5 ± 0.4

with a methylene bridge between the β- and γ-phosphorus atoms did not decrease the K_D for K^+ (Table III). This failure is not attributable merely to poor occupancy of the active site: the affinity for the analog was estimated in terms of competition toward ATP (Fig. 3: K_i = 3.8 mM). Thus with 0.5 mM analog occupancy was achieved comparable to that with the ATP and CTP concentrations used (K_m for ATP, 0.62 mM (Fig. 3); K_m for CTP, 2.8 mM (Fig. 4)).

Dimethylsulfoxide, although it decreases the $K_{0.5}$ for K^+ of the phosphatase activity, does not decrease the $K_{0.5}$ for K^+ of the ATPase activity [19]. In accord with this, dimethylsulfoxide did not decrease the K_D for K^+ in the presence of a phosphorylating nucleotide (Table III). Oligomycin, which

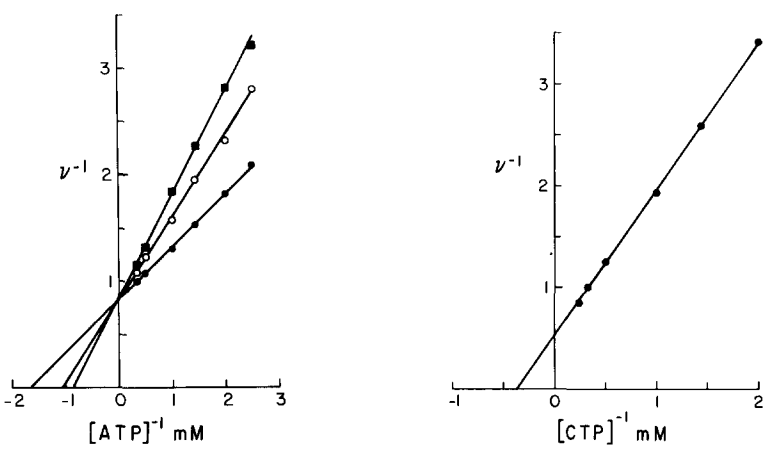

Fig. 3. Inhibition of the (Na^+ + K^+)-dependent ATPase by the β, γ-methylene analog of ATP. The enzyme preparation was incubated in 30 mM histidine · HCl · Tris (pH 7.8), 6 mM $MgCl_2$, 90 mM NaCl, 10 mM KCl, and the concentration of ATP indicated, in the absence (●) or presence of the β, γ-methylene analog of ATP, at a concentration of 2 (○) or 3 (■) mM.

Fig. 4. (Na^+ + K^+)-dependent CTPase activity. The enzyme preparation was incubated in the standard medium, except that CTP was substituted for ATP at the concentrations indicated. Velocities are expressed relative to that with 3 mM CTP defined as 1.0.

favors the E_1 form of the enzyme [2,22,23], had no measurable effect on the K_D for K^+.

The high-affinity K^+ sites demonstrable under conditions producing enzyme phosphorylation, e.g. the overall ATPase reaction, or the phosphatase reaction in the presence of Na^+ and a nucleotide, or in these inactivation experiments, are termed β sites.

Cation efficacy

Occupancy of the α and β sites can also be distinguished in terms of the relative efficacy of Li^+ as an analog of K^+ (Table IV). In comparison with other monovalent cations the efficacy of Li^+ at the α sites (V for the phosphatase activity) is far less than at the β sites (V for the ATPase activity). For the other cations no such disparity in efficacy, relative to K^+, was apparent although the range in affinity ($K_{0.5}$ for activation) for all the ions was quite broad at both classes of sites (Table IV). The failure of Li^+ as a substitute for K^+ in the phosphatase reaction has been noted previously [24] although the distinction between affinity and efficacy has not been drawn.

Unfortunately, the efficacy of Li^+ in promoting inactivation by F^- is so low that it could not be quantitated accurately. Such a poor effect, however, is consistent with the low efficacy of Li^+ at α sites.

In the absence of K^+, Na^+ can stimulate the phosphatase activity [4]; The $K_{0.5}$ is about 5 mM, but the relative V is only 0.04 (Table IV). Thus Na^+ differs from Li^+ in having an even lower efficacy at the α sites, although occupying the sites with a higher affinity.

Effects of pH

A further distinction between the α and β sites was demonstrable in terms of the effects of pH on affinity at each class of sites (Fig. 5). For the α sites, measured in terms of the phosphatase activity or K^+-stimulated inactivation by F^-, the affinity declined at higher pH values, with a calculated pK_a of about

TABLE IV

RELATIVE EFFICACIES AND AFFINITIES OF MONOVALENT CATIONS

V and $K_{0.5}$ were determined kinetically from incubations in the standard media, varying the concentration of KCl or the other cations substituted for it. In the case of Tl^+, the NO_3^- anion was substituted for Cl^- throughout. Data are presented ± S.D.; V is listed in units relative to the activity of KCl in the standard media.

Cation	Phosphatase activity		ATPase activity	
	V	$K_{0.5}$ (mM)	V	$K_{0.5}$ (mM)
Tl^+	1.14 ± 0.09	0.29 ± 0.05	1.01 ± 0.04	0.15 ± 0.05
Rb^+	1.20 ± 0.08	1.7 ± 0.4	1.01 ± 0.04	0.74 ± 0.15
K^+	1.10 ± 0.05	1.9 ± 0.3	1.03 ± 0.03	0.80 ± 0.12
Cs^+	1.02 ± 0.11	11 ± 2	1.04 ± 0.06	6.2 ± 1.9
Li^+	0.15 ± 0.03	36 ± 8	0.52 ± 0.10	11 ± 3
Na^+	0.04 ± 0.02	6 ± 2	—	—
NH_4^+*	1.13 ± 0.08	13 ± 3	1.32 ± 0.12	6.1 ± 1.8

* Data are from ref. 5, reproduced for comparison.

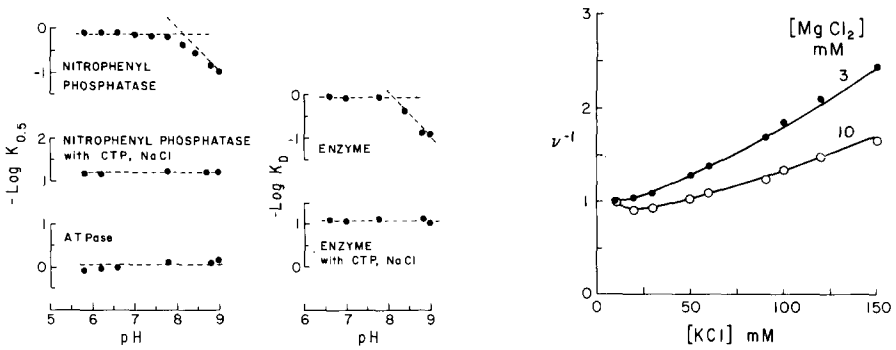

Fig. 5. Effects of pH on the $K_{0.5}$ and K_D for KCl. The $K_{0.5}$ for KCl was determined from Hill plots of enzymatic activity. K^+-dependent phosphatase activity was measured in the standard medium and in the standard medium containing in addition 0.3 mM CTP and 10 mM NaCl, with varying KCl concentrations and at the pH values shown. The $K_{0.5}$ for KCl for the $(Na^+ + K^+)$-dependent ATPase was measured similarly, altering only the KCl and the pH of the standard medium. The K_D for KCl was estimated as indicated in Fig. 2 and Tables I and III. In all cases the pH shown is the measured pH of the complete medium.

Fig. 6. Inhibition of the K^+-dependent phosphatase by KCl. Phosphatase activity was measured either in the standard medium (●), or in media containing 10 mM $MgCl_2$ (○), with the KCl concentrations indicated. Data are presented in the form of a Dixon plot. The lines in the figure were generated by substituting the parameters of Table V in Eqn 2.

8.1. By contrast, no such decrease in affinity at high pH was seen for the β sites, measured in terms of the ATPase activity or the phosphatase activity in the presence of nucleotide and Na^+, or the inactivation by F^- in the presence of nucleotide and Na^+.

At pH 8.6 the relative affinities of the other monovalent cations in the phosphatase reaction changed in concert with that of K^+:Tl^+, 1.1 mM; K^+, 7.9 mM; Rb^+, 7.5 mM; and Cs^+, approx. 50 mM. The K_i for Na^+ toward K^+ also increased proportionately, to 21 mM.

Inhibitory sites for K^+

Inhibition at high K^+ concentrations has been often noted with both the ATPase and phosphatase activities (e.g. see refs 5 and 25), and this has been attributed at least in part to competitions with other cations at their activating sites. However, since the enzyme when functioning as the sodium/potassium pump must discharge the transported ions with each reaction cycle it seems possible that release of the monovalent cations may exert a kinetic effect: retention of the cation at the discharge site would inhibit the enzyme. To investigate whether inhibition by high K^+ concentrations might result from occupancy of discharge sites as well as from competition at other cation sites, the phosphatase reaction was chosen for study to eliminate one such set of competition (with Na^+).

At concentrations of KCl greater than 30 mM inhibition becomes noticeable (Fig. 6). This is due in part to apparent competition with Mg^{2+}, since in the presence of 10 mM $MgCl_2$ such inhibition only becomes obvious at higher KCl concentrations. Nevertheless, the sharply increasing inhibition at still higher KCl concentrations cannot be fitted to a simple model with mutual

competition between K^+ and Mg^{2+} (in terms of the Hill equation with competitive interactions):

$$v = \frac{V}{\left\{1 + \left[\frac{K_1}{[K^+]}\left(1 + \frac{[Mg^{2+}]}{K_2}\right)\right]^{n}\right\}\left\{1 + \frac{K_3}{[Mg^{2+}]}\left(1 + \frac{[K^+]}{K_4}\right)\right\}} \quad (1)$$

This inhibition may be accomodated, however, by adding a third factor to the denominator, representing occupancy by K^+ of non-competitive inhibitory site(s) of low affinity, K_5, which may interact (when $n_2 \neq 1$):

$$v = \frac{V}{\left\{1 + \left[\frac{K_1}{[K^+]}\left(1 + \frac{[Mg^{2+}]}{K_2}\right)\right]^{n_1}\right\}\left\{1 + \frac{K_3}{[Mg^{2+}]}\left(1 + \frac{[K^+]}{K_4}\right)\right\}\left\{1 + \left(\frac{[K^+]}{K_5}\right)^{n_2}\right\}} \quad (2)$$

The superiority of Eqn 2 was demonstrated, following an empirical curve-fitting approach, using the generalized likelihood-ratio theorem [26]. Plausible values for K_1, K_2, K_3 and n_1 were selected (Table V) for Eqn 1 from the experimental data at low (<20 mM) KCl concentrations (Fig. 7). A range of values for K_4 was then examined to minimize the sum of the squared residuals between the logarithm of the observed velocities and the logarithm of the calculated velocities; "best" fit was with K_4 = 15 mM. Next, with the same values of K_1, K_2, K_3, and n_1 (since the two equations diverge markedly only at higher KCl concentrations) values for K_4 and K_5 were examined, again using the criterion of minimizing the sum of the squared residuals. The "best" fit was with K_4 = 70 mM and K_5 = 140 mM (Table V). With the generalized likelihood-ratio theorem, which tests the difference between a "full" and a "reduced" equation, the "full" equation (Eqn 2) was markedly superior ($p < 0.001$).

As can be seen, the kinetic parameters satisfy the data for the two $MgCl_2$ concentrations examined, both at low KCl concentrations (Fig. 7) where K^+/Mg^{2+} competitions dominate, and at high concentrations (Fig. 6) where the inhibitory sites are preeminent.

TABLE V

KINETIC PARAMETERS FOR ACTIVATION AND INHIBITION

Kinetic parameters for Eqn 2 were obtained by an empirical curve-fitting technique. These values were used to generate the lines in Figs 6—9. Units of K_1—K_5 are mM; n_1 and n_2 are dimensionless.

Parameter	With K^+	With Tl^+	
		Case I	Case II
n_1	1.45	1.6	1.6
n_2	1.0	1.0	1.4
K_1	1.35	0.20	0.20
K_2	5.0	5.0	5.0
K_3	0.75	0.75	0.75
K_4	70	40	40
K_5	140	12	18

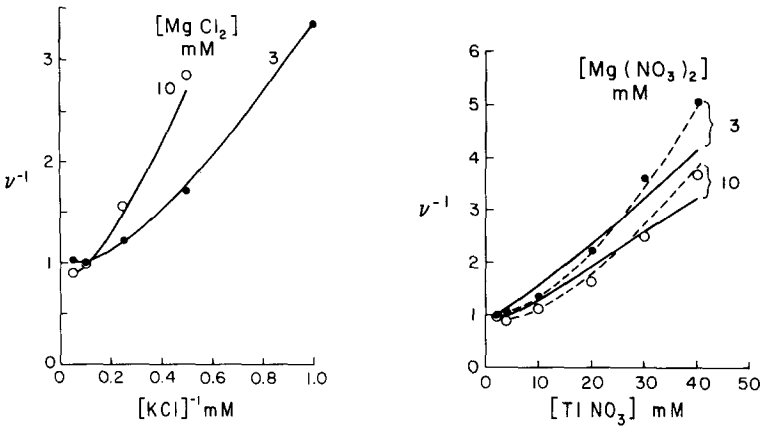

Fig. 7. K^+-dependent phosphatase activity at two $MgCl_2$ concentrations. These data represent identical experiments to those in Fig. 6 except that they cover a lower range of KCl concentrations; here data are presented in double reciprocal form. Again, the lines represent the parameters of Table V substituted in Eqn 2.

Fig. 8. Inhibition of Tl^+-dependent phosphatase activity by $TlNO_3$. These experiments correspond to those of Fig. 6 except that $TlNO_3$ was substituted for KCl and the NO_3^- anion throughout for Cl^- (because of the far greater solubility of $TlNO_3$ compared to TlCl). The solid lines were generated by substituting the parameters for $TlNO_3$ of Table V, with $n_2 = 1.0$, in Eqn 2, and the dashed lines the parameters with $n_2 = 1.4$.

A different explanation for the inhibition at high KCl concentrations might lie in considerations of ionic strength effects. Addition of 100 mM LiCl or choline chloride (in the presence of 20 mM KCl and 3 mM $MgCl_2$) also inhibited: 44 and 27%, respectively. Such inhibition might represent: (i) an effect of ionic strength or the anion concentration; (ii) competition with K^+ by a cation of lesser efficacy (from the relative affinities and efficacies of Li^+ and K^+ such inhibition can be calculated to be less than 15%); (iii) low specificity of the inhibitory site; or a combination of these.

An alternative approach to these issues is to examine inhibition by $TlNO_3$, which not only activates the phosphatase at lower concentrations than K^+, but also inhibits in the concentration range where K^+ activates: it is thus difficult to attribute the inhibition by Tl^+ to effects of ionic strength. (KNO_3 was no more inhibitory than KCl so the greater potency cannot be ascribed to NO_3^-.) As with inhibition by KCl the inhibition by $TlNO_3$ rose sharply at higher concentrations (Fig. 8), and clearly cannot be fitted to a model with only Tl^+/Mg^{2+} competitions. By the curve-fitting approach a reasonable correspondence with the data is obtained with plausible kinetic parameters (Table V) at two $MgCl_2$ concentrations (Figs 8 and 9). However, the Tl^+ inhibition seems to increase even more rapidly than this model predicts; a better fit can be obtained for the higher $TlNO_3$ concentrations if cooperative interactions are postulated between the inhibitory sites ($n_2 > 1.0$), just as is proposed between the activating cation sites ($n_1 > 1.0$). Such cooperative interactions may be incorporated into the equation for inhibition by KCl as well (with concomitant changes in K_4 and K_5), but in the range of KCl concentrations examined the data do not require it.

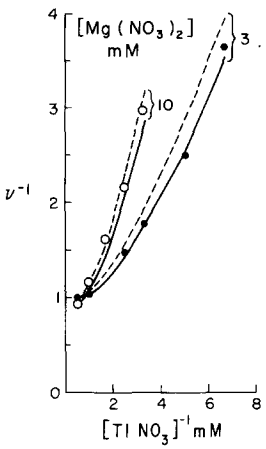

Fig. 9. Tl^+-dependent phosphatase activity at two $Mg(NO_3)_2$ concentrations. These experiments correspond to those of Fig. 7 except that $TlNO_3$ was substituted for KCl and NO_3^- for Cl^-. The solid and dashed lines correspond to those of Fig. 8.

Since the inhibition seen with KCl and $TlNO_3$ might be due to a contaminating impurity, the inhibition was also examined in the presence of 0.1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetate, which is an effective chelator of trace-metal inhibitors of this enzyme [27]; no difference was observed.

No decrease in K_5 was seen in the presence of phlorizin or dimethylsulfoxide, although both decreased K_1.

Discussion

K^+ sites of the (Na^+ + K^+)-dependent ATPase are here categorized into three classes, initially in terms of affinity, although further examination yields other distinguishing criteria as well. Affinities for K^+ have been estimated by two approaches: (i) in terms of the activation kinetics of the enzymatic reactions, and (ii) through K^+-accelerated inactivation of the enzyme by F^-. This latter approach permits estimation of a dissociation constant K_D, rather than an apparent affinity in terms of $K_{0.5}$; moreover, it also allows estimation of affinity with ligand states of the enzyme in which the standard kinetic approach is inapplicable (e.g. absence of substrate). The procedure is entirely comparable to that using K^+-dependent inactivation by Be^{2+} [6], but has a major advantage for the present studies of not suffering from chelation of inactivator by the nucleotides studied. The sites controlling inactivation are clearly on the enzyme, and the identification with the kinetically determined K^+ sites can be supported both in terms of comparable affinities and of analogous sensitivity to modifiers (e.g. phlorizin, dimethylsulfoxide).

The α sites, characterized by these techniques as having a moderate affinity for K^+ (K_D is approx. 1 mM), are thus found on the "free" enzyme (absence of ligands other than Mg^{2+}, which is required for inactivation). Sites of similar affinity also function in the K^+-dependent phosphatase activity, which reflects the terminal hydrolytic steps of the overall ATPase reaction, apparently bypass-

ing the initial Na^+-dependent steps [4,24]. The identification as α sites of both the sites controlling enzyme inactivation and those activating the phosphatase reaction is strengthened by further correspondence between their properties: a similar K_i for Na^+, a decreased affinity for K^+ at high pH with a pK_a of about 8.1, and the lesser efficacy of Li^+ when occupying these sites. Thus for the phosphatase activity a plausible reaction scheme would be:

$$
\begin{array}{l}
{}_{\alpha}E \xrightarrow[K^+]{MgXP} \rightarrow {}_{K\alpha}EXP \rightarrow \rightarrow \quad {}_{\alpha}E + X + P_i \\
\qquad\qquad\qquad\qquad\qquad\qquad\quad K^+ \downarrow\uparrow \\
\qquad\qquad\qquad\qquad\qquad\qquad\quad {}_{K\alpha}E
\end{array}
$$

where XP represents the phosphatase substrate and the subscript to E represents the pertinent class of sites available to K^+. No firm data on the order of addition of K^+ and XP are available. Although it is apparent that K^+ must bind before XP hydrolysis, release of K^+ might occur at any step thereafter; unfortunately, there is no information on the properties of the α-sites until the terminal reappearance of the free enzyme. Release of hydrolysis products seems to follow an ordered mechanism [28], but these considerations cannot yet be linked to K^+ binding.

Rega et al. [29] have demonstrated that the K^+ sites activating the phosphatase reaction in erythrocytes are accessible from the external medium, just as are the K^+ sites activating the ATPase reaction (here termed β sites). These α sites are not the external Na^+ sites of the enzyme from which Na^+ is discharged after transport since their affinity for Na^+ is too high (K_i approx. 5 mM) to function effectively as the external sites for releasing Na^+ into the extracellular fluid (the $K_{0.5}$ for Na^+ on the exterior, from Na^+/Na^+ exchange studies [30], seems to be >100 mM). Moreover, the affinity of these sites for both Na^+ and K^+ is incompatible with the internal Na^+ sites governing enzyme phosphorylation (where K_D for Na^+ is approx. 2 mM [10]; $K_{0.5}$ for Na^+ with the ATPase is approx. 2 mM when the K^+ concentration is extrapolated to zero [5,7]; K_i for K^+ as competitor is approx. 12 mM in terms of the $K_{0.5}$ for Na^+ as a function of K^+ concentration [5,7]).

In the presence of nucleotides the apparent affinity for K^+ is decreased in terms of both the phosphatase reaction (Robinson, J.D., unpublished) and inactivation (Table III), so long as enzyme phosphorylation does not occur; however, the change in affinity is only several-fold.

By contrast, under conditions when enzyme phosphorylation does occur the affinity for K^+ markedly increases: high affinity β sites become apparent. These are the sites seen for the overall ATPase reaction ($K_{0.5}$ is approx. 0.1 mM as the competing Na^+ concentration is extrapolated to zero [5,7]), for the phosphatase reaction in the presence of nucleotides and Na^+ ($K_{0.5}$ is approx. 0.1 mM [4,7]), and in the inactivation experiments with phosphorylating agent and Na^+ (K_D is approx. 0.1 mM; Table III). These values are also in accord with the activation of the sodium/potassium pump in erythrocytes by external K^+ in the absence of competing Na^+ [31]. Again, the calculated inhibition by Na^+ is comparable: K_i of about 40 mM for the pump [31], and 35 mM for the ATPase [5], estimated in terms of the increase in $K_{0.5}$ as Na^+ concentration increases.

The appearance of the β sites would seem to require actual phosphorylation, beyond any allosteric effects of nucleotide binding, not only because of the additional requirement for Na^+ (essential for phosphorylation) but also because the non-phosphorylating analogs (Table III and ref. 32) are unable to produce the effect whereas structurally dissimilar agents such as acetyl phosphate (Table III) and nitrophenyl phosphate [32], under conditions where they phosphorylate the enzyme, can. It would seem likely that β sites appear through a conformational change in pre-existing α sites [6,7], although the continuous existence of α sites cannot be ruled out.

Unlike the α sites, the β sites do not decrease their affinity for K^+ at higher pH values (Fig. 5). Moreover, at these sites the efficacy of Li^+, relative to K^+, is far higher than at the α sites (Table IV). This latter observation is consistent with the marked increase in Li^+-stimulated phosphatase activity when Na^+ and a phosphorylating nucleotide are added [24].

These considerations may be incorporated into a plausible scheme for the appearance and participation of the β sites in the overall ATPase reaction:

$$_{\alpha}E \xrightarrow[Na^+]{Mg\ ATP} \rightarrow {}_{\beta}E - P \overset{+K^+}{\rightarrow} {}_{K\beta}E - P \overset{-K^+}{\rightarrow} \rightarrow {}_{\alpha}E + ADP + P_i$$

Although the high affinity β sites can be assigned to the E-P intermediate, the specific order of release of ADP, P_i, Na^+, and K^+ from the enzyme (and pump) remains unspecified.

Low affinity inhibitory sites, γ sites, are demonstrable in kinetic studies (Figs 6—9). There is no direct evidence linking them to the internal sites from which K^+ is discharged into the cytoplasm, although their affinity is consistent with such a role. It may be noted that in vivo there is no physiological necessity for cation specificity at such sites.

In an important paper Post et al. [11] proposed that in the course of K^+ transport by the ATPase a rate-limiting step can be the release of this cation, that there is an intermediate conformation in which the K^+ sites are occluded, and that ATP is physiologically important to effect the release of this tightly bound K^+. This formulation is based in part on considerations of ATP/K^+ antagonisms in terms of bindings to the enzyme, and also on comparisons of the relative efficacies of Rb^+ and Li^+ in (i) promoting enzyme dephosphorylation, and (ii) hindering phosphorylation. They argued that Rb^+ inhibits rephosphorylation (relative to Li^+) because of its retention at the discharge sites.

Garrahan and Garay [33] have recently argued, on the basis of the sodium/potassium pump kinetics, against an "occluded conformation" occupying a significant temporal fraction of the reaction cycle. Beyond this, consideration of the data of Post et al. [11] suggests that the inhibitory effects of Rb^+ are more likely exerted at α sites rather than at discharge sites.

That increasing ATP concentrations decreased the apparent affinity for K^+, and increasing K^+ that for ATP, was first indicated in kinetic studies [3]. More direct demonstrations, by Nørby and Jensen [34] and Hegyvary and Post [35], showed an effect of K^+ on the K_D for ATP binding to the enzyme. In these experiments Mg^{2+} and Na^+ were absent so that enzyme phosphorylation could not occur, and thus the effect of K^+ was not mediated through β sites.

Furthermore, the K^+ concentrations producing half-maximal changes in K_D were in the range of the α sites and far lower than would be expected of discharge sites.

The disparity between Li^+ and Rb^+ can also be accounted for in terms of α and β sites: concentrations of Li^+ and Rb^+ equipotent for dephosphorylation, an action at β sites, would not be equipotent at α sites (Table IV): Rb^+ would be expected to exert a greater effect. Prior phosphorylation of the enzyme is not necessary for cations to act at the α sites, in terms of the phosphatase activity, F^- inactivation, or decreased K_D for ATP. Finally the available data do not suggest that physiological levels of ATP can decrease the affinity of α sites enough to make them appropriate for discharge, where a $K_D > 100$ mM would be expected [7].

A model to explain the inhibition of phosphorylation [11] by K^+ (or Rb^+) through occupancy of the α sites would be:

$$
\begin{array}{ccccccccc}
{}_{\alpha}E_{\gamma} & \overset{MgATP}{\rightleftharpoons} & {}_{\alpha}E_{\gamma}\cdot MgATP & \overset{Na^+}{\rightarrow} & {}_{\beta}E_{\gamma}-P & \overset{K^+}{\rightarrow} & {}_{K\beta}E_{\gamma}-P & \overset{Pi}{\dashrightarrow} & {}_{\alpha}E_{\gamma K} \rightarrow {}_{\alpha}E_{\gamma} + K^+ \\
K^+ \downarrow\uparrow & & K^+ \downarrow\uparrow & & & & & & \\
{}_{K\alpha}E_{\gamma} & \overset{MgATP}{\rightleftharpoons} & {}_{K\alpha}E_{\gamma}\cdot MgATP & & & & & &
\end{array}
$$

where the subscripts to E indicates the class of site available for K^+; Na^+ and K^+ may bind at the same time at their own sites [1,4—9]. The lower loop is extraneous to the overall reaction scheme, but insofar as binding to α-sites favors such steps K^+ will be inhibitory*. K^+ discharge is proposed to occur, following transport through a "gate" or "oscillating pore", from low affinity discharge sites [6,7], perhaps the γ sites demonstrated here.

Recently, Grisham and Mildvan [36] proposed an intriguing model for the pump function of this ATPase in which a change in the ionization state of a phosphate at the active site determines the binding of first Na^+ and then K^+. The data presented here on pH and affinity for K^+ of the overall ATPase reaction (Fig. 5) would not appear to be consistent with such a mechanism.

Acknowledgements

I wish to thank Miss Grace Marin for meticulous technical assistance and Mr Edward Matyas for computer programming. I am indebted to Dr Paul Sheehe for assistance with the statistical analyses. This work was supported by U.S. Public Health Service research grant NS-05430.

References

1 Skou, J.C. (1971) in Current Topics in Bioenergetics, (Sanadi, D.R., ed.), Vol. 4, pp. 357—398, Academic Press, New York

2 Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727—756

* In vivo the α sites, situated on the external membrane surface [28], would be occupied chiefly by Na^+, in light of the ionic composition of the extracellular fluid and the relative affinities for K^+ and Na^+. Since Na^+ competitively overcomes the effects of K^+ on the apparent affinity for ATP, [35] no inhibition would then occur.

3 Robinson, J.D. (1967) Biochemistry 6, 3250—3258
4 Robinson, J.D. (1969) Biochemistry 8, 3348—3355
5 Robinson, J.D. (1970) Arch. Biochem. Biophys. 139, 17—27
6 Robinson, J.D. (1973) Arch. Biochem. Biophys. 156, 232—243
7 Robinson, J.D. (1974) Ann. N. Y. Acad. Sci., in press
8 Hoffman, P.G. and Tosteson, D.C. (1971) J. Gen. Physiol. 58, 438—466
9 Garay, R.P. and Garrahan, P.J. (1973) J. Physiol. 231, 297—325
10 Robinson, J.D. (1974) FEBS Lett. 38, 325—328
11 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540
12 Ulrich, F. (1963) Biochem. J. 88, 193—206
13 Atkins, G.L. (1973) Eur. J. Biochem. 33, 175—180
14 Opit, L.J., Potter, H. and Charnock, J.S. (1966) Biochim. Biophys. Acta 120, 159—161
15 Yoshida, H., Nagai, K., Kamei, M. and Nakagawa, Y. (1968) Biochim. Biophys. Acta 150, 162—164
16 Lahiri, A.K. and Wilson, I.B. (1971) Mol. Pharmacol. 7, 46—51
17 Penzotti, S.C., Jr. and Titus, E.O. (1972) Mol. Pharmacol. 8, 149—158
18 Robinson, J.D. (1969) Mol. Pharmacol. 5, 584—592
19 Robinson, J.D. (1972) Biochim. Biophys. Acta 274, 542—550
20 Tobin, T., Baskin, S.I., Akera, T. and Brody, T.M. (1972) Mol. Pharmacol. 8, 256—263
21 Israel, Y. and Titus, E. (1967) Biochim. Biophys. Acta 139, 450—459
22 Fahn, S., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1882—1889
23 Robinson, J.D. (1971) Mol. Pharmacol. 7, 238—246
24 Robinson, J.D. (1970) Arch. Biochem. Biophys. 139, 164—171
25 Green, A.L. and Taylor, C.B. (1964) Biochem. Biophys. Res. Commun. 14, 118—123
26 Mood, A.M. and Graybill, F.A. (1963) Introduction to the Theory of Statistics, pp. 275—327, Mc Graw-Hill, New York
27 Specht, S.C. and Robinson, J.D. (1973) Arch. Biochem. Biophys. 154, 314—323
28 Robinson, J.D. (1970) Biochim. Biophys. Acta 212, 509—511
29 Rega, A.F., Garrahan, P.J. and Pouchan, M.I. (1970) J. Membrane Biol. 3, 14—25
30 Glynn, I.M. and Lew, V.L. (1969) 54, 289S—305S
31 Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. 192, 175—188
32 Robinson, J.D. (1973) Biochim. Biophys. Acta 321, 662—670
33 Garrahan, P.J. and Garay, R.P. (1974) Ann. N. Y. Acad. Sci. in press
34 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
35 Hegyvary, G. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
36 Grisham, C.M. and Mildvan, A.S. (1974) J. Biol. Chem. 249, 3187—3197