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# SUBSTRATE SITES OF THE ($Na^+ + K^+$)-DEPENDENT ATPase

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## Summary

Kinetic studies on a rat brain ($Na^+ + K^+$)-dependent ATPase (EC 3.6.1.3) preparation demonstrated high-affinity sites for ATP, with a $K_m$ near 1 $\mu$m, and low affinity sites for ATP, with a $K_m$ near 0.5 mM. In addition, the dissociation constant for ATP at the low affinity sites was approached through the ability of ATP to modify the rate of photo-oxidation of the enzyme in the presence of methylene blue; a value of 0.4 mM was obtained. The temperature dependence of the $K_m$ values in these two concentration ranges also differed markedly, and the estimated entropy of binding was +27 cal/degree per mol at the high affinity sites, whereas it was —20 cal/degree per mol at the low-affinity sites. Moreover, the relative affinities of various congeners of ATP as competitive inhibitors at these sites were distinctly different. Substrate kinetics of the $K^+$-dependent phosphatase reaction of the enzyme indicated an interaction at the low-affinity sites for ATP: ATP, ADP, CTP, and the [$\beta$-$\gamma$]-imido analog of ATP all competed with $K_i$ values near those for the ATPase reaction at the low affinity sites. Conversely, the $K_m$ for nitrophenyl phosphate as a substrate for the phosphatase reaction was near its $K_i$ as a competitor at the low-affinity sites of the ATPase reaction. These observations are incorporated into a reaction scheme with two classes of substrate sites on a dimeric enzyme, manifesting diverse enzymatic and transport characteristics.

## Introduction

The ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been convincingly identified with the sodium pump that transports $Na^+$ and $K^+$ across the cell membrane, and the properties of enzyme and pump have been extensively scrutinized [1—4]. From this wealth of information, however, seeming discrepancies and contradictions continue to emerge. For example, kinetic studies on the enzyme using ATP concentrations in the range of those required for optimal hydrolytic activity reveal a $K_m$ of the order of 0.1 mM,

whereas studies of ATP binding indicate a dissociation constant three orders of magnitude lower [2—5]. Moreover, the $Na^+$-dependent ADP/ATP exchange reaction of the enzyme appears to have a marked specificity for ATP, whereas certain other reactions show greater tolerance for alternative substrates [6—8]. Most formulations of the reaction sequence for this ATPase continue to portray a single class of substrate sites [2—4], but considerations of such issues suggest the possibility that more than one class of substrate sites participate in enzymatic processes [5,9—13].

This paper is concerned with characterizing some properties of the substrate interactions in terms of comparative kinetic studies with ATP in both the micromolar and millimolar concentration ranges, and of extending estimates of the dissociation constant for ATP to the millimolar range. In addition, relative affinities for a series of ATP congeners are compared in these concentration ranges. The data are interpreted in terms of two distinct classes of substrate sites, differing markedly in affinity for ATP, in thermodynamic parameters for binding, and in relative substrate specificity. This formulation, in turn, necessitates revisions in the overall reaction scheme to accommodate these as well as other recent observations on both enzyme and pump properties.

## Methods and Materials

The enzyme preparation was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described [14].

($Na^+ + K^+$)-dependent ATPase activity was measured in terms of the liberation of $P_i$ using a spectrophotometric method as previously described [14], or, in experiments in which tracer quantities of [$\gamma$-$^{32}$P] ATP were added, as the liberation of $^{32}P_i$. In the latter experiments the $^{32}P_i$ was extracted according to the procedure described by Neufeld and Levy [15], and the radioactivity measured with a liquid scintillation counter. The standard incubation media contained, in addition to the specified concentrations of Tris · ATP and $MgCl_2$, 30 mM Tris/histidine · HCl (pH 7.8), 90 mM NaCl and 10 mM KCl. Activity in the absence of $Na^+$ and $K^+$, which averaged only a few percent of the ($Na^+ + K^+$)-dependent activity under optimal conditions, was measured concurrently, and was substracted from the total activity in the presence of $Na^+$ and $K^+$ to give the ($Na^+ + K^+$)-dependent activity [14]. Because of variations in the absolute activity of different enzyme preparations, velocities are expressed relative to the ($Na^+ + K^+$)-dependent ATPase activity of a concurrent control incubation at 37°C, with 3.5 mM $MgCl_2$ and 3.0 mM ATP, 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 described previously [16]. The standard media contained, in addition to the specified concentrations of Tris · *p*-nitrophenyl phosphate and $MgCl_2$, 30 mM Tris/histidine · HCl (pH 7.8) and 10 mM KCl. 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 [16], and was subtracted from the total activity to give the $K^+$-dependent activity. As with the ATPase, velocities are expressed relative to $K^+$-dependent phosphatase activity

of concurrent incubations at 37°C, in the presence of 3 mM *p*-nitrophenyl phosphate and 3 mM $MgCl_2$, defined as 1.0.

Photo-oxidation of the enzyme in the presence of methylene blue was performed using slight modifications of the method previously described [17]. The enzyme preparation was incubated at 0°C in the presence of 30 mM Tris · HCl (pH 7.8) and 3 μM methylene blue, plus other additions as noted, under illumination by a 100-W tungsten bulb at a distance of 20 cm. After incubation for the specified time the light was switched off and the medium was diluted with an equal volume of 0.25 M sucrose. Assay of the residual activity was performed immediately, under dim illumination, at 37°C in the standard medium with 3 mM ATP and 3.5 mM $MgCl_2$; the final dilution of the original inactivating medium was 8-fold. Extent of inactivation was assessed relative to corresponding controls containing the same additions but kept in the dark during the inactivating incubation. Illumination in the absence of methylene blue did not produce inactivation.

ATP, ADP, CTP, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., and the [β-γ]-imido analog of ATP and [$\gamma$-$^{32}$P]ATP from ICN Isotope and Nuclear Division. Substrates and analogs were converted to Tris salts, as previously described [14].

The data presented are averages of four or more experiments performed in duplicate or triplicate. The straight lines of the kinetic plots were fitted by eye.

## Results

*Apparent affinities for ATP*

When ($Na^+ + K^+$)-dependent ATPase activity was measured in terms of the release of $^{32}P_i$ from [$\gamma$-$^{32}$]ATP, so that ATP hydrolysis could be measured at very low substrate concentrations, a biphasic Lineweaver-Burk plot was obtained (Fig. 1). At higher ATP concentrations an apparent $K_m$ of 0.5 mM can be calculated from the plot (Table I), in accord with earlier determinations with this enzyme preparation using a spectrophotometric method to measure $P_i$ release [5,14]. From the data at the lower ATP concentrations an apparent $K_m$ of about 1 μM can be estimated (Fig. 1, Table I), approaching values for the dissociation constant for ATP measured in binding studies [18—20]. A similar biphasic Lineweaver-Burk plot was previously reported by Kanazawa et al. [21].

These results are considered here as suggestive evidence for "high-affinity sites" and "low-affinity sites" for ATP. At the "low-affinity sites" kinetic studies [5,22] indicate that Mg · ATP is the true substrate, although this may not necessarily be the case at the "high-affinity sites". However, in the experiments described here sufficient $MgCl_2$ was present so that the concentration of Mg · ATP was essentially equivalent to the concentration of total ATP.

The plot (Fig. 1) also shows that the maximal velocity extrapolated from the data representing the low concentrations of ATP was about 1/10 that with the high ATP concentrations.

As the incubation temperature was reduced from 37°C the apparent $K_m$ measured at the higher ATP concentrations (at the "low-affinity sites") markedly decreased (Fig. 2). By contrast, the apparent $K_m$ measured at the lower ATP concentrations (at the "high-affinity sites") was little affected by changes in

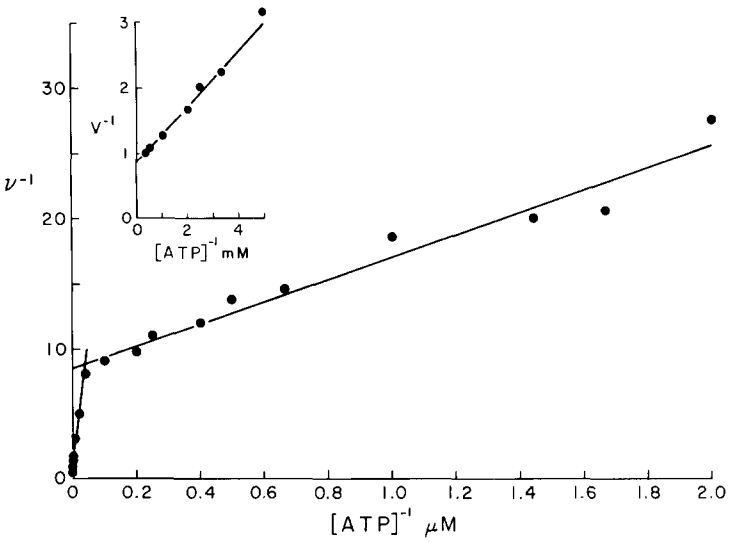


Fig. 1. Substrate kinetics of the $(Na^+ + K^+)$-dependent ATPase. Enzymatic activity was measured in terms of $^{32}Pi$ release from [$\gamma$-$^{32}P$] ATP. Incubations were at 37°C in the standard media (see Methods) with the concentration of ATP indicated. The $MgCl_2$ concentrations were 500 μM greater than the ATP concentrations; keeping the $MgCl_2$ concentrations 50 μM greater than the ATP concentrations produced qualitatively similar results. Data are presented in double-reciprocal Lineweaver-Burk form; in the inset the data with the higher concentrations of ATP are replotted.

temperature. Assuming that these $K_m$ values reflect the dissociation constants from high- and low-affinity sites on the enzyme, then the free energies of binding, $\Delta G^0$, would be about −8.5 and −4.7 kcal, respectively. Similarly, dramatic differences in enthalpy and entropy of binding at the two classes of sites can be estimated (Table II).

Neufeld and Levy [15] described a $Na^+$-stimulated/$K^+$-inhibited ATPase activity, demonstrable at low ATP concentrations, in a partially purified $(Na^+ + K^+)$-dependent ATPase preparation. In accord with their observations, $Na^+$-dependent ATPase activity in this preparation, measured in the absence of $K^+$,

TABLE I

RELATIVE AFFINITIES OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

Values are taken from the experiments shown in Figs. 1, 4, 5, and 8.

| Agent | $K_m$ or $K_i$ values | | $K_D$ (mM) |
|---|---|---|---|
| | at low ATP concentrations (μM) | at high ATP concentrations (mM) | from methylene blue-induced photo-oxidation |
| ATP | 1 | 0.5 | 0.4 |
| [β-γ]-imido analog | 3 | 0.4 | — |
| ADP | 8 | 0.3 | 0.5 |
| CTP | 70 | 2.0 | 4.0 |
| nitrophenyl phosphate | — | 5.0 | 7.0 |

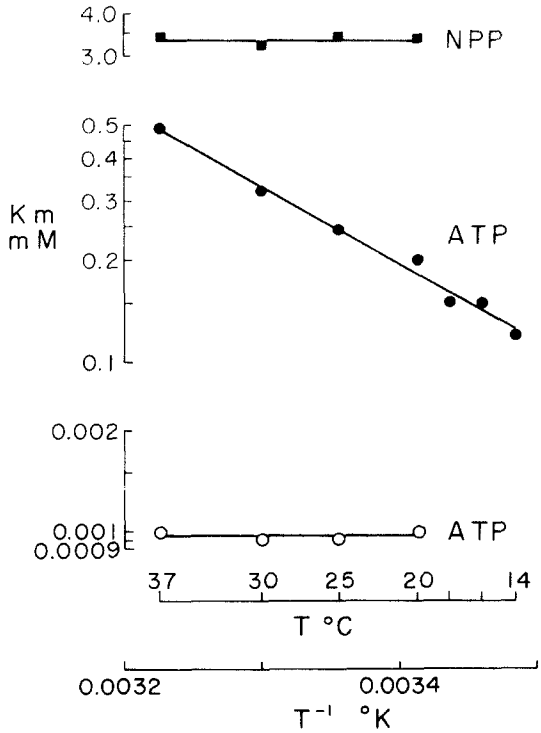


Fig. 2. Effect of temperature on the substrate kinetics of the $(Na^+ + K^+)$-dependent ATPase and $K^+$-dependent phosphatase reactions. The $K_m$ for ATP was determined, as in Fig. 1, over a series of incubation temperatures. In analogous experiments the $K_m$ for nitrophenyl phosphate, as a substrate for the $K^+$-dependent phosphatase reaction, was also measured over a series of incubation temperatures, in the standard media with nitrophenyl phosphate concentration ranging from 5.0 to 0.7 mM; the $MgCl_2$ concentrations were 500 $\mu$M greater than the nitrophenyl phosphate concentrations. The pH of the buffers used was adjusted to 7.8 at the incubation temperature used. Data are presented in the form of an Arrhenius plot.

had an apparent $K_m$ of about 0.3 $\mu$M (Fig. 2), somewhat lower than the $K_m$ for the $(Na^+ + K^+)$-ATPase activity at the "high-affinity sites". Moreover, the Lineweaver-Burk plot for this $Na^+$-ATPase activity appeared to be linear: there was no indication of a second $K_m$ at higher ATP concentrations. Neufeld and Levy [15] also presented data on $(Na^+ + K^+)$-ATPase activity that suggest a biphasic Lineweaver-Burk plot.

TABLE II

THERMODYNAMIC PARAMETERS OF ATP BINDING

Estimates of the thermodynamic parameters at 37°C were made from the data of Fig. 2, assuming that the $K_m$ measured is a fair approximation of the dissociation constant; a uniform over- or underestimate of the true dissociation constant has relatively little effect on $\Delta H^0$ and $\Delta S^0$ (e.g. if the true dissociation constant is 0.2 $\mu$M at the "high-affinity sites", $\Delta S^0$ is changed to +31 cal/degree per mol).

| Sites | $\Delta G^\circ$ (kcal) | $\Delta H^\circ$ (kcal) | $\Delta S^\circ$ (cal/degree per mol) |
|---|---|---|---|
| "High-affinity sites" | −8.5 | 0 | +27 |
| "Low-affinity sites" | −4.7 | −10.8 | −20 |

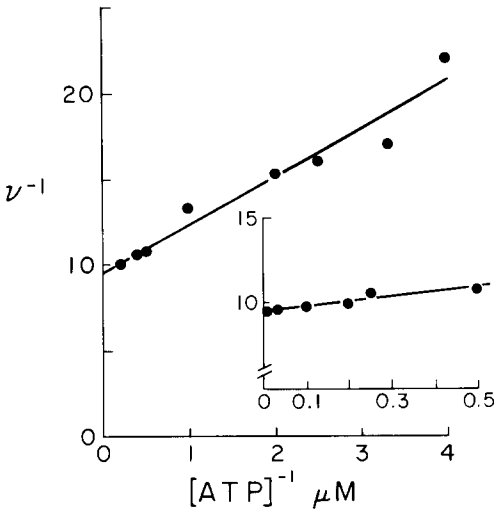


Fig. 3. Substrate kinetics of the $Na^+$-dependent ATPase reaction. Experiments were performed and the data are presented as in Fig. 1, except that the standard media contained 20 mM NaCl rather than 90 mM NaCl plus 10 mM KCl. Essentially the same data were obtained in the presence of 100 mM NaCl. In the inset experiments extending the ATP concentration to 0.1 mM are presented.

*Competitors to ATP*

To distinguish further between the two classes of substrate sites of the ($Na^+$ + $K^+$)-dependent ATPase, the effects of possible competitors were examined. In the presence of low concentrations of ATP Dixon plots were consistent with competitive inhibition (Fig. 4) with ADP, CTP, and the [β-γ]-imido analog

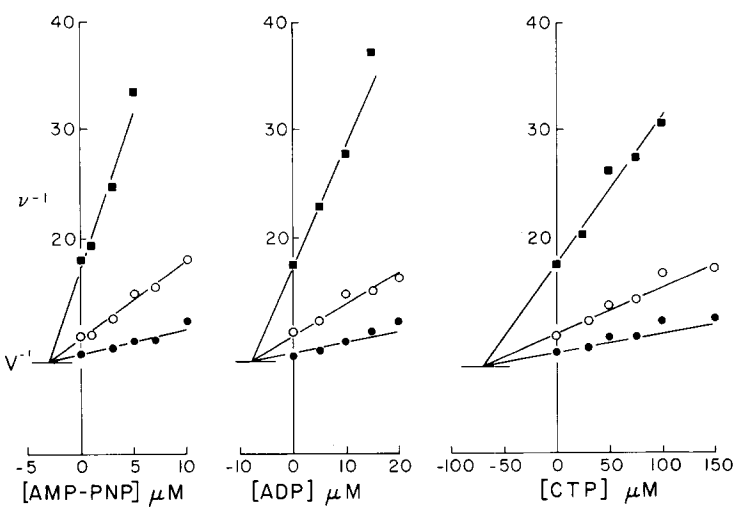


Fig. 4. Competitive inhibition of the ($Na^+$ + $K^+$)-dependent ATPase activity. ATPase activity was measured as in Fig. 1, at 37°C in the standard media containing the concentrations of [β-γ]-imido analog of ATP, ADP, and CTP shown, and with 10 (●), 3 (○), and 1 (■) μM ATP. The $MgCl_2$ concentrations were 500 μM greater than the sum of ATP and competitor. Data are presented in the form of Dixon plots, with the reciprocal of the maximal velocity for the low concentration range (see Fig. 1) shown by the horizontal line.

of ATP (abbreviated AMP-PNP in the figures). The estimated $K_i$ values ranged from 3 to 70 $\mu$M (Table I); straight lines were drawn for the Dixon plots although more complex interactions could not be rules out with the data available. In the presence of high concentrations of ATP the Dixon plots again were consistent with competitive inhibition (Fig. 5), but the range of $K_i$ values was considerably narrower (Table I). These compounds also inhibited the $Na^+$-ATPase activity, with $K_i$ values similar to those at the "high-affinity sites" of the ($Na^+ + K^+$)-dependent ATPase reaction (data not presented).

Nitrophenyl phosphate, even at concentrations 500 times that of ATP, did not detectably inhibit ($Na^+ + K^+$)-dependent ATPase activity measured with 1 $\mu$M ATP. However, with ATP concentrations in the range of the "low-affinity sites" nitrophenyl phosphate appeared to be a competitive inhibitor (Fig. 5). In this case the $K_i$ was near the $K_m$ for nitrophenyl phosphate (3.3 mM) as a substrate for the $K^+$-dependent phosphatase reaction of the enzyme.

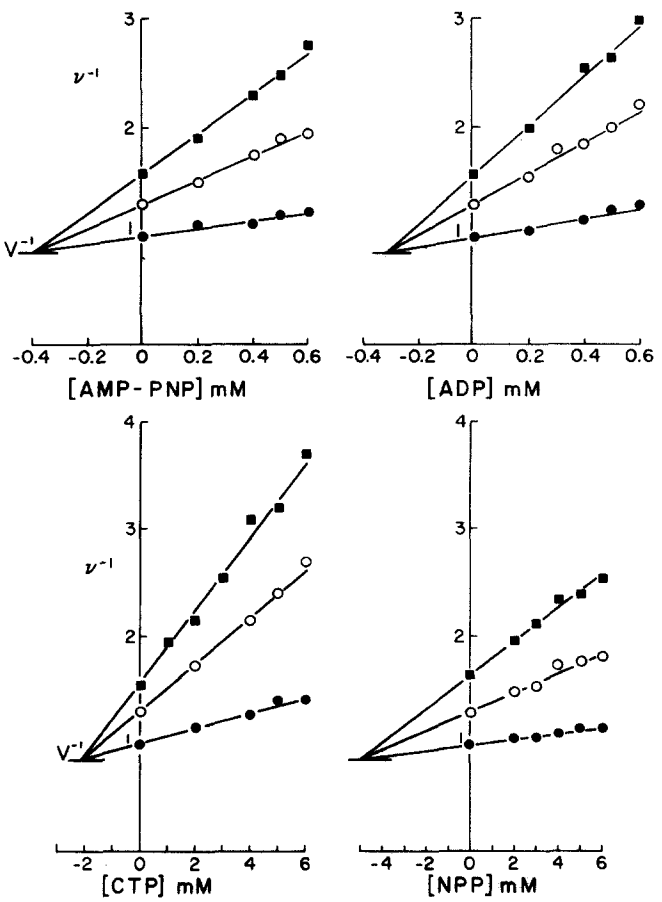


**Fig. 5. Competitive inhibition of the ($Na^+ + K^+$)-dependent ATPase activity. Experiments were performed and the data are presented as in Fig. 4, except that the ATP concentrations were 3 (●), 1 (○), and 0.6 (■) mM, and the horizontal line indicating the reciprocal of the maximal velocity is for the high concentration range of ATP.**

*Substrate kinetics of the $K^+$-dependent phosphatase reaction*

($Na^+$ + $K^+$)-dependent ATPase preparations exhibit $K^+$-dependent phosphatase activity with substrates such as nitrophenyl phosphate, and this phosphatase activity has been interpreted as a reflection of the terminal $K^+$-dependent hydrolytic steps of the overall ATPase reaction [2—4]. Nevertheless, it is not clear precisely what sites on the enzyme are available to the phosphatase substrates. One approach to this issue would be to compare the abilities of various ATPase substrates and competitors as inhibitors of the phosphatase reaction. Dixon plots with ATP, ADP, CTP, and the [$\beta$-$\gamma$]-imido analog of ATP all are consistent with competitive inhibition (Fig. 6). Moreover, the $K_i$ values (Table III) are in accord with $K_i$ values as competitors of the ATPase reaction with the higher concentrations of ATP (at the "low-affinity sites"), and the $K_i$ for ATP is of the same magnitude as the $K_m$ for ATP at the "low-affinity sites" (Table I).

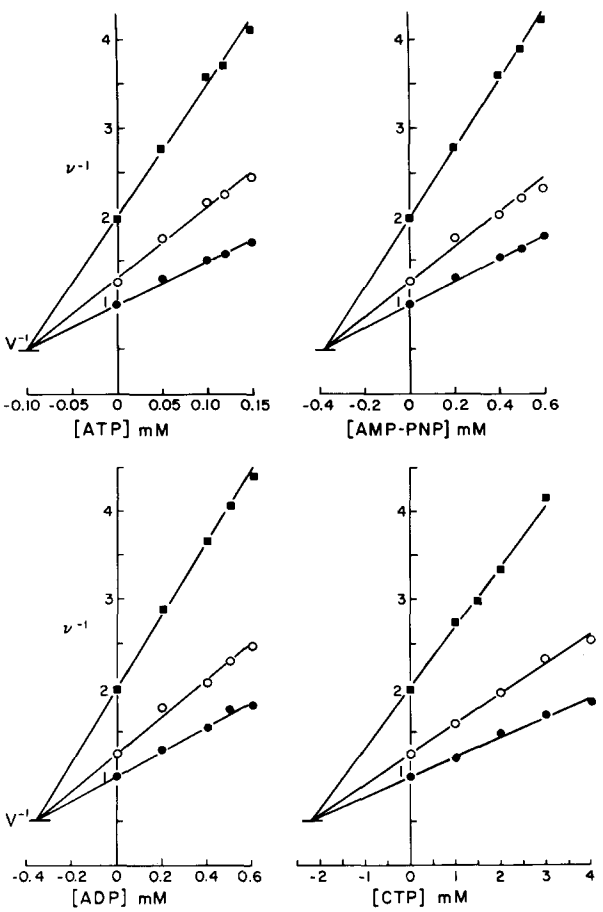


Fig. 6. Competitive inhibition of the $K^+$-dependent phosphatase activity. Data are presented as in Fig. 4 from similar experiments measuring phosphatase activity at 37°C in the standard media (see Methods), with 3 (●), 2 (○), and 1 (■) mM nitrophenyl phosphate.

TABLE III

KINETIC PARAMETERS OF THE $K^+$-DEPENDENT PHOSPHATASE REACTION

Values are taken from Fig. 6.

| Parameter | | Value (mM) |
|---|---|---|
| $K_m$ for nitrophenyl phosphate | | 3.3 |
| $K_i$ for: | ATP | 0.1 |
| | [β-γ]-imido analog | 0.4 |
| | ADP | 0.35 |
| | CTP | 2.0 |

Low concentrations of certain nucleotides (e.g. ATP, CTP), below the $K_m$ for the "low-affinity sites", can stimulate the phosphatase reaction measured with low $K^+$ concentrations [16,23], suggesting an interaction of nucleotide at the "high-affinity sites" while the phosphatase substrate is hydrolyzed at the "low-affinity sites". The [β-γ]-imido analog of ATP was unable to substitute for ATP or CTP, although its affinity at both sets of sites is intermediate, in accord with the proposal that phosphorylation by nucleotides is necessary to effect the stimulation [23,24].

When the incubation temperature was reduced the $K_m$ for nitrophenyl phosphate did not decrease sharply as it did for ATP at the "low-affinity sites" (Fig. 2). Perhaps the changes in $K_m$ for ATP at the "low-affinity sites" with changes in temperature represent an interaction between enzyme and ATP that nitrophenyl phosphate cannot achieve, even when binding at the same sites. Furthermore, nitrophenyl phosphate need not occupy precisely the same loci when acting as a competitor to the ATPase activity.

*Methylene blue-sensitized photo-oxidation*

Attempts to demonstrate a second class of ATP sites by direct measurements of binding to the enzyme are inherently more difficult because of the postulated lower affinity. An alternative approach to estimating the dissociation constant for ATP is suggested by the ability of ATP to protect against photo-oxidation of the enzyme in the presence of methylene blue [17]. By analogy with previous studies of protection against inactivation by specific ligands [25,26], the following model may be proposed:

$$E \cdot ATP \overset{ATP}{\rightleftharpoons} E \xrightarrow{h\nu} E_{in}$$

in which the free enzyme, E, is susceptible to photo-oxidation (forming inactive enzyme, $E_{in}$), whereas the enzyme-ATP complex is resistant. In accord with this model, incubations of the enzyme with 3 μM methylene blue in the presence of light lead to inactivation of the enzyme (as measured by subsequent assay), which followed a first-order time course (Fig. 7). Addition of ATP slowed the rate of inactivation in a concentration-dependent fashion. With this model [25,26]:

$$\frac{k - k'}{k} = \frac{1}{1 + \frac{K_D}{[ATP]}}$$

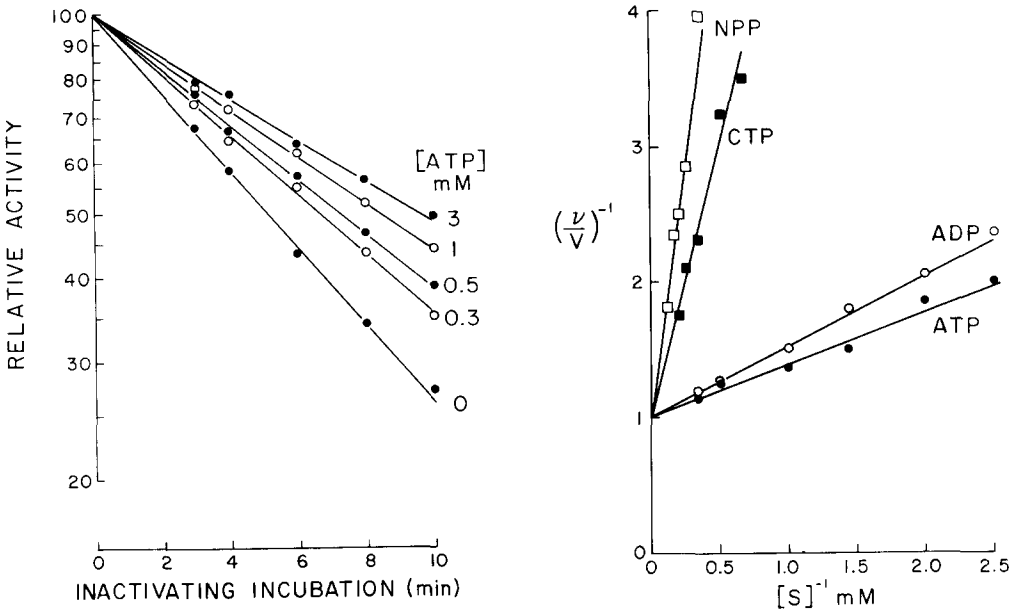


Fig. 7. Photo-inactivation of the enzyme. The enzyme was inactivated by incubation at 0°C, under strong illumination, for the times indicated, in the presence of 30 mM Tris · HCl (pH 7.8) and 3 μM methylene blue; the residual ($Na^+ + K^+$)-dependent ATPase activity was then measured, as described under Methods. This residual activity is presented relative to corresponding controls incubated in the dark. Inactivation in the presence of ATP, at the concentrations listed, is also shown. $MgCl_2$ was also included at a concentration 500 μM greater than ATP; in the absence of ATP these concentrations of $MgCl_2$ did not measurably affect inactivation.

Fig. 8. Effects of substrates and analogs on photo-inactivation of the enzyme. Pseudo-first-order rate constants for inactivation were obtained as in Fig. 7, in the absence and presence of the agents shown, at the concentrations indicated. As described in the text, the reciprocal of the concn. of agent is plotted against the reciprocal of $(k - k')/k$, defined as $v$, which is normalized by dividing by the maximal rate of inactivation, indicated by $V$.

where $k$ is the pseudo-first-order rate constant for inactivation in the absence of ATP, and $k'$ that in the presence of a specific concentration of ATP; $K_D$ is the dissociation constant for ATP at the sites influencing inactivation. For convenience, and to emphasize the analogy with the Michaelis-Menten equation, $(k - k')/k$ is here termed $v$, and a plot of $v^{-1}$ against $[ATP]^{-1}$ will therefore lead to a linear plot with an intersection on the abscissa at $-K_D^{-1}$ (Fig. 8). These experiments thus support the existence of "low-affinity sites" for ATP, with a $K_D$ near the $K_m$ for ATP seen at higher ATP concentrations, and also near the $K_i$ for ATP as a competitor to the phosphatase reaction. It was not technically possible to examine the effects of low ATP concentrations to determine if this plot is also biphasic.

ADP, CTP, and nitrophenyl phosphate also protected against photo-oxidation, and when comparable experiments were performed (Fig. 8) the dissociation constants for these compounds approximated the values previously determined for $K_m$ or $K_i$ at the "low-affinity sites" (Table I).

## Discussion

The experiments described here support models for the ($Na^+ + K^+$)-dependent ATPase with two classes of ATP sites: high-affinity sites, comparable to those measured in binding studies [18—20] and also studied in experiments on enzyme phosphorylation [27]; and low affinity sites, as measured in kinetic studies with levels of ATP near those found in vivo [5,14,22]. Proposals for these latter sites have included modulating roles in the hydrolytic process and participation in complex half-of-the-sites-active reaction mechanisms [5,9—13,26]. Beyond the differences in dissociation constant for ATP, the data show that the high-affinity and low-affinity sites also differ in their thermodynamic parameters, notably the entropy of binding, and in their relative affinities for various congeners of ATP.

Although these two classes of sites could exist on the same polypeptide, several lines of evidence suggest that the ATPase exists as a dimer of two large catalytic subunits (mol. wt. approx. 100 000): radiation-inactivation experiments indicate a molecular weight of about 250 000 [2]; the catalytic subunits can be cross-linked in situ in pairs [28]; and binding studies with ATP and ouabain reveal under certain experimental conditions a 2 : 1 stoichiometry with phosphorylating sites [3]. Thus it is tempting to envisage the high-affinity ATP site on one unit of the dimer, with the low-affinity site on the other monomer. (Purified ATPase preparations also contain glycopeptides with a molecular weight roughly half that of the catalytic polypeptide [2—4]; consequently, the total ensemble of the ATPase probably consists of several subunits of these different protomers.)

The substrate sites for the phosphatase activity seem to correspond with the low-affinity sites for ATP. This interpretation allows ATP at the high-affinity sites to stimulate the phosphatase reaction at the low-affinity sites. An alternative explanation in terms of one class of substrate sites requires that the enzyme first be phosphorylated by ATP and undergo a conformational change representing the activation; after dephosphorylation the enzyme then catalyzes several cycles of the phosphatase reaction before relaxing to its pre-activation state [9,29].

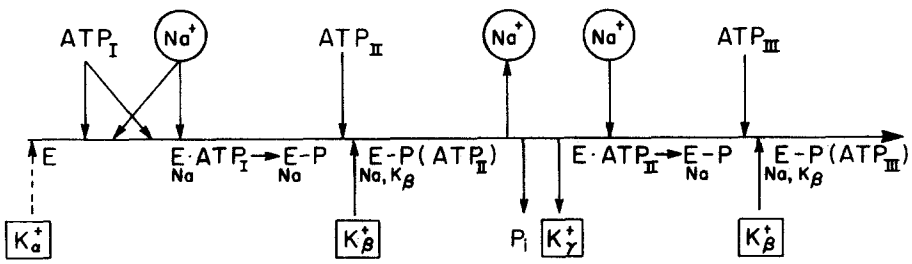


Fig. 9. Proposed reaction scheme for the ($Na^+ + K^+$)-dependent ATPase. The horizontal line represents the time course of the reaction, beginning with enzyme in the absence of substrate. Vertical arrows pointing toward this line indicate additions of substrates and activators, arrows pointing away from the line the release of products (the release of ADP is not indicated: see text). The subscripts to ATP distinguish between the sequential additions of ATP to the enzyme. The subscripts to $K^+$ indicate at which class of sites $K^+$ is bound [30]: moderate-affinity $\alpha$-sites, activating high-affinity $\beta$-sites, and low-affinity discharge $\gamma$-sites.

From these considerations a reaction scheme with two classes of participating ATP sites can be constructed (Fig. 9) with the following characteristics:

(1) The free enzyme contains sites for $K^+$ of moderate affinity, termed $\alpha$-sites [30], coexisting with activating sites for $Na^+$. Binding of $K^+$ to these $\alpha$-sites decreases the apparent affinity for ATP [5,18,19,31], making the $K_m$ for the ($Na^+ + K^+$)-ATPase higher than that for the $Na^+$-ATPase (Figs. 1 and 3). $K^+$ at these sites could pull the reaction to the left in $K^+$-stimulated transfer of phosphate from the enzyme to form ATP [32,33] and in $K^+$-stimulated ADP/ATP exchange [34]. With ATP concentrations sufficient to fill the low affinity sites, the successive cycles of enzymatic activity shown in the scheme do not again pass through the free enzyme form; thereafter the high-affinity $\beta$-sites for $K^+$ [30] would be demonstrable, as recent studies on the sodium pump indicate [13].

(2) The order of binding of ATP to the high affinity sites and of $Na^+$ to its activating sites is probably random: neither ligand appreciably affects the binding of the other [9,13,25,26,31].

(3) The $E_1/E_2$ nomenclature is not included because the original criteria have been variously modified. Although successive forms of enzyme-phosphate intermediate probably exist, the transition seems not to be achieved by a cyclic addition of free $Mg^{2+}$ [5,35,36], as originally proposed [37].

(4) The reversible release of ADP from the phosphorylated enzyme, representing the $Na^+$-dependent ADP/ATP exchange reaction [6,37], is not specified but probably precedes the addition of ATP to the low-affinity sites. Here the ADP/ATP exchange is a function of the high-affinity sites. Thus the greater specificity of this reaction for ATP [6], in comparison with the overall ATPase reaction, reflects the differing specificity for congeners of ATP at these two classes of sites. This formulation can also account for $Mg^{2+}$ dependence of the ADP/ATP exchange [37]. With high concentrations of ATP the low $MgCl_2$ levels permit ADP/ATP exchange because the MgATP concentration is below the $K_m$ at the low affinity sites; conversely, high $MgCl_2$ levels inhibit the exchange because the Mg · ATP concentration is now sufficient to fill the low-affinity sites and thereby pull the reaction to the right.

(5) $Na^+$-ATPase activity, measured in the absence of $K^+$, would also represent occupancy of only the high affinity sites (Fig. 3). The sodium pump, which normally effects a $Na^+/K^+$ exchange, can in the absence of $K^+$ effect a net transport of $Na^+$ ("uncoupled sodium pump") at a far slower rate [3]. The affinity for ATP of the uncoupled pump is high (approx. 1 $\mu$M) without evidence for low-affinity sites, whereas ATPase activity of this preparation in the presence of $Na^+$ and $K^+$ has a $K_m$ consistent with the low-affinity sites [12]. Nevertheless, at least part of the "$Na^+$-ATPase" activity measured might represent the ($Na^+ + K^+$)-ATPase reaction activated by tightly-bound $K^+$. Goldfarb and Rodnight [38] found that a small content of $K^+$ tightly bound to their enzyme preparation effectively stimulated the ($Na^+ + K^+$)-ATPase in the presence of low ATP concentrations. Although the enzyme preparation used here was extensively washed [14], residual tightly-bound ions might influence this or other cation-sensitive functions.

(6) The sodium pump also catalyzes a $Na^+/Na^+$ exchange in the absence of $K^+$, which apparently represents reversible interactions between ADP and the phos-

phorylated enzyme, without release of inorganic phosphate [3]. This requires that $Na^+$ be discharged from the enzyme before $P_i$. Recent studies by Taniguchi and Post [39] on reversing that ATPase reaction show that, after formation of an enzyme-phosphate complex, high concentrations of $Na^+$ (consistent with its binding to the discharge sites of the pump) can pull the reaction to the left to form ATP.

(7) The second ATP is shown binding to the low-affinity sites before $K^+$ binds, in accord with observations that ATP concentrations near the $K_m$ at the low-affinity sites affect the apparent affinity for $K^+$ at the $\beta$-sites [14]. On the other hand, $K^+$ may influence the interactions of ATP at the low affinity sites, since in the absence of $K^+$ ATP has no demonstrable effect on either the $Na^+$-ATPase activity or the uncoupled sodium pump (above). Because ATP at the low-affinity sites can dramatically alter the reaction properties, extrapolations from studies at low ATP concentrations to ATP hydrolysis with physiological levels of ATP must be guarded.

(8) $K^+$ release from its discharge sites should follow release of $P_i$, for $K^+$ profoundly inhibited phosphorylation of the enzyme by $P_i$ [39].

(9) In the absence of $Na^+$ the pump can catalyze a $K^+/K^+$ exchange [3]. ATP is required for this reaction at concentration consistent with occupancy of the low-affinity sites [11]; however, enzyme phosphorylation is not required since non-phosphorylating analogs of ATP can be substituted [40]. It seems likely that $K^+/K^+$ exchange represents a shuttling over the reaction pathway between $K^+$ binding to discharge ($\gamma$) and acceptance ($\beta$) sites.

(10) After release of $P_i$ and $K^+$, and subsequent binding of $Na^+$, an attractive possibility is the phosphorylation of the enzyme by the ATP molecule that was bound second. This would then be followed by the binding of a third ATP molecule to a low-affinity site. Considerations of symmetry suggest that the binding to the ATP sites alternate between the two dimeric units as the reaction proceeds.

(11) The model depicts $Na^+$ release after $K^+$ binding, in contrast to some earlier transport schemes that were equivalent to bisubstrate ping-pong models (i.e. $Na^+$ is released before $K^+$ binds) [2—4]. Recent evidence against ping-pong models includes the demonstration of coexisting $Na^+$ and $K^+$ sites [26,41] and pump kinetics inconsistent with bisubstrate ping-pong reactions, i.e. non-parallel Lineweaver-Burk plots as one substrate is varied at different fixed levels of the other [42—44]. Nevertheless, neither set of arguments rules out mechanisms in which $Na^+$ sites become $K^+$ sites in the course of the transport cycle. With a dimeric transport system one monomer could be in the $Na^+$ form while the other is in the $K^+$ form, with affinities then reversing at the next cycle; thus $Na^+$ and $K^+$ sites coexist for the total enzyme, even though not present on the same monomer. The kinetic evidence is also inconclusive, for the reaction is clearly not a bisubstrate one. With three reactants, even if two follow the ping pong scheme, parallel Lineweaver-Burk plots need not occur [45].

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