Biochimica et Biophysica Acta, 341 (1974) 232–247

BBA 67174

NUCLEOTIDE AND DIVALENT CATION INTERACTIONS WITH 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 October 4th, 1973)

SUMMARY

With a brain microsomal $(Na^+ + K^+)$-dependent ATPase preparation (EC 3.6.1.3) the apparent K_m for the MgATP complex was 0.48 mM. Free ATP was a competitive inhibitor with a K_i of 4.8 mM, whereas the apparent affinity for free Mg^{2+}, determined by a Be^{2+} inactivation technique, was 0.8 mM. Free Mg^{2+} was also a weak non-competitive inhibitor to MgATP, with a K_i of about 40 mM. Mg^{2+} antagonized the effects of the modifier oligomycin, and oligomycin reduced the apparent affinity for Mg^{2+}, consistent with their favoring alternative allosteric states of the enzyme. Inhibition by $CaCl_2$ was "mixed", in keeping with competitive inhibition by CaATP toward MgATP together with competition by Ca^{2+} at Na^+-sites, also demonstrated. With the associated K^+-dependent nitrophenylphosphatase activity $CaCl_2$ was a conventional competitor toward $MgCl_2$. Examination of the effects of free Mg^{2+} indicated that reaction schemes proposing a cyclical addition to and release from the enzyme of Mg^{2+} must incorporate a cyclical change in affinity of at least four orders of magnitude. An alternative scheme is suggested with multiple substrate sites of differing affinity, only half of which may be hydrolytically active at a given time.

INTRODUCTION

The physiologically important relationship between the $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) and the active transport of Na^+ and K^+ across cell membranes has attracted much attention to this enzyme. Yet despite numerous detailed investigations the complexities of the reaction process continue to obscure such primary considerations as the nature of the enzyme–substrate interactions. This report is concerned with several aspects of these interactions. First: the early proposal [1] that MgATP is the true substrate of the enzyme has recently been questioned [2, 3]. This point may be in part semantic since it is clear that both nucleotide and divalent cation are required. At issue, however, is the nature of the site(s) for Mg^{2+} and ATP: is there merely one ternary complex of Mg–ATP–enzyme, or do separate sites exist, and if so, what are their characteristics and how are these sites normally filled? Second: the conventional reaction scheme for the ATPase [4, 5] includes the compulsory binding of an additional Mg^{2+} during the hydrolytic

sequence, with an obligatory release of this Mg^{2+} at a subsequent stage. The plausibility of such a formulation depends in part on what the necessary characteristics of such auxiliary Mg^{2+}-sites must be. Third: the enzyme is known to exist in several functionally distinguishable states [4, 5], and among the major determinants of these states are reported to be Mg^{2+} [4–6], Ca^{2+} [3], and the antibiotic oligomycin [4, 7, 8]. Thus interactions among these agents are pertinent both to the cation-nucleotide sites and to the regulatory cation site cited above.

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 [9].

$(Na^+ + K^+)$-dependent ATPase activity was measured in terms of the production of P_i, as previously described [9]. The standard medium contained 50 mM Tris–HCl (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/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 [9], 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*-nitrophenylphosphate, as previously described [10]. The standard medium contained 50 mM Tris–HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenylphosphate (as the Tris salt), 20 mM KCl, and the enzyme preparation (0.1 mg protein/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 [10], 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 binding of Mg^{2+} to the enzyme was also approached in terms of Mg^{2+}-dependent inactivation by Be^{2+}, as previously described in detail [11]. In brief, these experiments involved determining the pseudo first-order rate constants for enzyme inactivation as a function of Mg^{2+} concentration, by means of initial incubations at 37 °C of enzyme (0.5 mg protein/ml), 50 mM Tris–HCl (pH 7.8), 50 μM $BeCl_2$, 10 mM KCl, and a range of $MgCl_2$ concentrations (plus other additions as specified). These inactivating incubations were terminated by adding 4 vol. of an incubation medium (with contents adjusted to provide final concentrations equivalent to the standard medium), and the residual activity then measured during brief incubations. Changes in activity during the latter incubations were negligible since the inactivation is essentially irreversible and the added NaCl blocks further inactivation.

ATP, ADP, and nitrophenylphosphate were purchased from Sigma Chemical

Co. as the sodium salts, and converted to the Tris salts. All solutions were made in water that had been 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. Concentrations of nucleotide–metal complexes were calculated using the following values of dissociation constants from O'Sullivan and Perrin [12]: MgATP = $1.36 \cdot 10^{-5}$ M; MgADP = $2.5 \cdot 10^{-4}$ M; CaATP = $3.22 \cdot 10^{-5}$ M. For Mg–nitrophenylphosphate the dissociation constant [10] used was $5.9 \cdot 10^{-3}$ M. Cubic equations were solved by computer.

RESULTS

MgATP as substrate

When Mg^{2+} and ATP were varied together at a 1:1 molar ratio in a range about cellular levels in vivo [13], the $(Na^+ + K^+)$-dependent ATPase activity seemingly followed the Michaelis–Menten relationship (Fig. 1), with an apparent K_m (designated

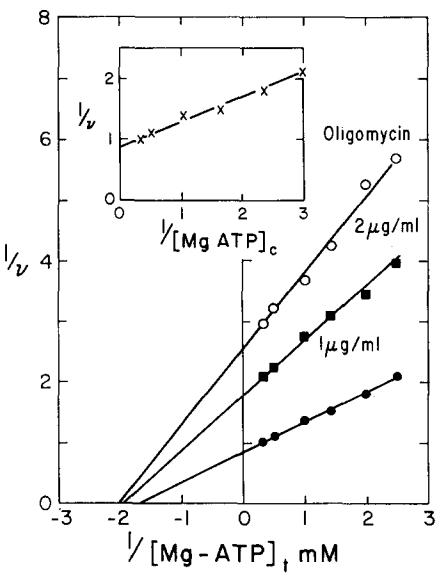

Fig. 1. Effects of ATP and Mg^{2+} on the $(Na^+ + K^+)$-dependent ATPase. The enzyme preparation was incubated in the standard medium (see Methods and Materials) but with the concentrations of ATP and $MgCl_2$ (at a 1:1 molar ratio) shown. Parallel experiments were performed in the presence of 1 μg/ml (■—■) and 2 μg/ml (○—○) oligomycin. Data are presented in the form of a double reciprocal Lineweaver–Burk plot. In the inset the calculated MgATP complex is plotted against velocity in the Lineweaver–Burk form.

here "K_m") of 0.58 mM (Table I). If, instead of the total Mg^{2+} and ATP concentrations, the concentrations of the calculated MgATP complex were plotted (Fig. 1, inset) then the same relationship was seen, although the "K_m" for the complex was 0.48 mM.

On the other hand, if either Mg^{2+} or ATP was varied at a fixed concentration

TABLE I

KINETIC PARAMETERS FOR ATP, ADP, AND $MgCl_2$

Values are taken from Figs 1–6, with calculations by standard formulae [15].

Conditions	Apparent kinetic parameter	
	"K_m" (mM)	"K_i" (mM)
$MgCl_2$ and ATP varied together	0.58	
ATP varied with 3 mM $MgCl_2$	0.68	
$MgCl_2$ varied with 3 mM ATP	0.80	
Excess free ATP		4.8
Excess free Mg^{2+}		40.0
MgADP		0.30

(3 mM) of the other, then the measured "K_m" was higher (Figs 2 and 3; Table I). In this case, at concentrations from 2 mM downward the calculated concentration of the MgATP complex is equal to that of the varied component, within 1 %.

To determine whether the higher "K_m" for MgATP in these latter cases resulted from inhibition by excess free Mg^{2+} and free ATP, the effects of these were examined. Free ATP appeared to be a competitive inhibitor toward MgATP (Fig. 4), as earlier reported [14]. It was, however, an extremely weak inhibitor with a "K_i" of 4.8 mM.

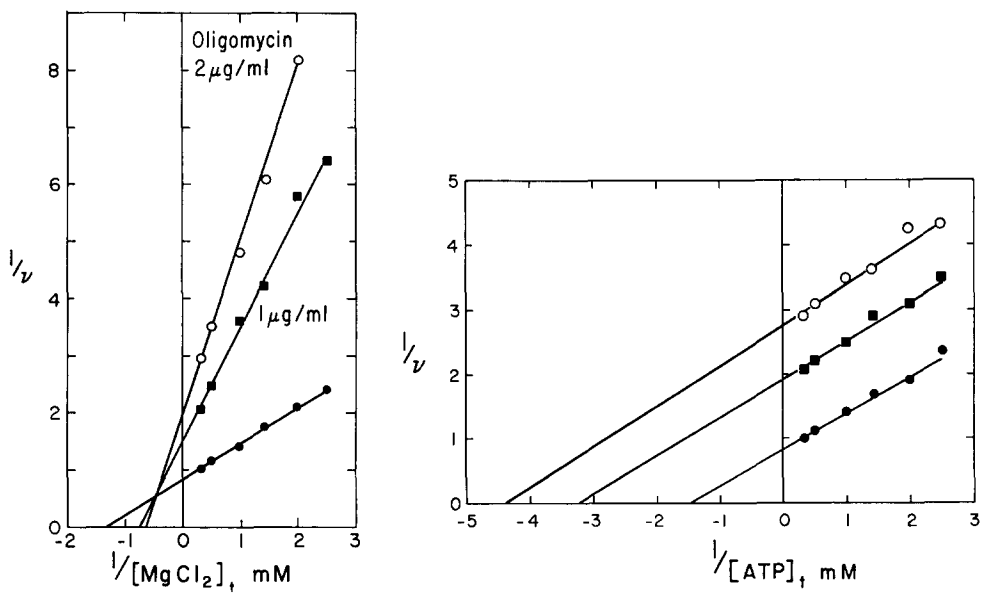

Fig. 2. Effects of Mg^{2+} on the (Na^+ + K^+)-dependent ATPase. Experiments were performed as in Fig. 1, except that $MgCl_2$ was varied in the presence of 3 mM ATP, in the absence (●—●) or presence of oligomycin: 1 μg/ml (■—■), or 2 μg/ml (○—○).

Fig. 3. Effects of ATP on the (Na^+ + K^+)-dependent ATPase. Experiments were performed as in Fig. 1, except that ATP was varied in the presence of 3 mM $MgCl_2$, in the absence (●—●) or presence of oligomycin: 1 μg/ml (■—■), or 2 μg/ml (○—○).

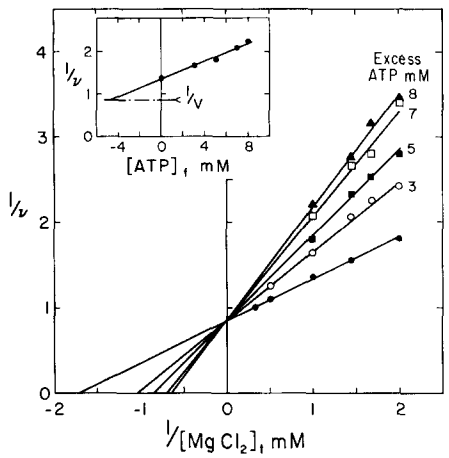

Fig. 4. Effects of excess ATP on the (Na^+ + K^+)-dependent ATPase. Experiments were performed as in Fig. 1 except that $MgCl_2$ was varied with ATP in the presence of equimolar ATP (●—●), or so that the total ATP concentration was greater than the total $MgCl_2$ by 3 mM (○—○), 5 mM (■—■), 7 mM (□—□), or 8 mM (▲—▲). In the inset calculated concentrations of free ATP are plotted against the reciprocal of the velocity in the presence of 1 mM total $MgCl_2$, in the form of a Dixon plot [15], showing the intersection with a horizontal line equal to the reciprocal of the maximal velocity, $1/V$.

When a competitor is varied with the substrate so that their molar sum is constant, as in Fig. 3, then the double reciprocal plot is still linear, although the measured "K_m" is obviously distorted.

Excess Mg^{2+} appeared here to be a non-competitive inhibitor toward MgATP (Fig. 5), although previously reported to be an uncompetitive inhibitor [14]. Other studies [6, 10] have shown that increased levels of divalent cations lowered the ap-

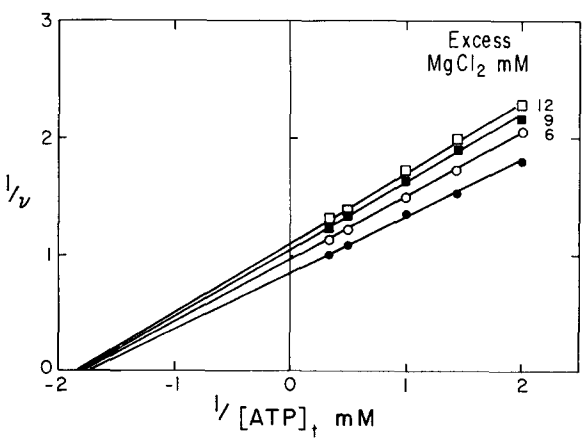

Fig. 5. Effects of excess Mg^{2+} on the (Na^+ + K^+)-dependent ATPase. Experiments were performed as in Fig. 1 except that ATP was varied with $MgCl_2$ in the presence of equimolar $MgCl_2$ (●—●), or so that the total $MgCl_2$ concentration was greater than the total ATP by 6 mM (○—○), 9 mM (■—■), or 12 mM (□—□).

parent affinity for Na^+ and K^+, which could account for the inhibition. In any case, free Mg^{2+} was an extremely weak inhibitor, with a "K_i" for non-competitive inhibition of about 40 mM.

Effects of oligomycin

Oligomycin is a well-documented modifier of this enzyme, reducing the ($Na^+ + K^+$)-dependent hydrolytic activity and increasing the Na^+-dependent ADP/ATP exchange activity [4, 7, 8]. Inhibition of the ATPase activity as a function of substrate concentration was, however, particularly complex. When ATP was varied at a fixed concentration of Mg^{2+}, adding oligomycin produced a family of nearly parallel lines (Fig. 3), suggesting uncompetitive inhibition, as previously noted [7]. However, when Mg^{2+} and ATP were varied together oligomycin produced a family of lines intersecting near the abscissa (Fig. 1), suggesting non-competitive inhibition, whereas when Mg^{2+} was varied with 3 mM ATP a pattern of "mixed" inhibition [15] resulted (Fig. 2).

These effects of oligomycin may be best visualized by considering what happened when either Mg^{2+} or ATP or both were reduced from initial values of 3 mM $MgCl_2$ and ATP, the experimental point common to Figs 1–3. Lowering the total ATP concentration to 0.5 mM (Fig. 3) reduces the MgATP complex to essentially that value, but also markedly increases the free Mg^{2+} concentration (Table II). Correspondingly, the velocity in the absence of oligomycin was halved, but in the presence of oligomycin, the velocity was reduced proportionately far less (30%). The result of this lesser inhibition at lower substrate concentrations is a pattern suggesting uncompetitive inhibition (Fig. 3). On the other hand, when the Mg^{2+} concentration was reduced to 0.5 mM, leaving the ATP concentration unchanged, then the concentration of MgATP is the same as above (Table II). In this case, however, the free Mg^{2+} is 0.003 mM rather than 2.5 mM. Oligomycin then inhibited proportionately more than with 3 mM ATP and Mg^{2+} (74% versus 66%), resulting in a pattern of "mixed" inhibition (Fig. 2). Finally, when both Mg^{2+} and ATP were reduced to 0.5 mM the concentration of MgATP was nearly the same as in the above two cases, but the concentration of free Mg^{2+} was intermediate (0.076 mM): similarly, the proportional inhibition by oligomycin was intermediate, being essentially the same as with 3 mM ATP and Mg^{2+}, and producing a pattern resembling non-competitive inhibition (Fig. 1).

TABLE II

DEPENDENCE OF OLIGOMYCIN INHIBITION ON Mg^{2+}

Values are from Figs 1–3; concentrations of the MgATP complexes are calculated from the dissociation constants [12].

$[MgCl_2]_t$ (mM)	$[ATP]_t$ (mM)	$[MgATP]_c$ (mM)	$[Mg^{2+}]_f$ (mM)	$[ATP]_f$ (mM)	Activity oligomycin		Inhibition (%)
					−	+	
3.0	3.0	2.805	0.195	0.195	1.00	0.34	66
3.0	0.5	0.497	2.503	0.003	0.52	0.23	56
0.5	0.5	0.424	0.076	0.076	0.55	0.19	65
0.5	3.0	0.497	0.003	2.503	0.47	0.12	74

Inhibition by ADP

ADP has previously been reported to be a competitive inhibitor (toward ATP) of the enzyme [14, 16]. Hexum et al. [14] considered this in terms of product inhibition, proposing that ADP was released after P_i. However, the active ADP/ATP exchange reaction suggests that ADP should be released before the breakdown of E–P. Consequently, it seemed of interest to re-examine this point in the current context.

To avoid inhibition due to competition with ATP for available Mg^{2+} the effects of ADP were examined in the presence of excess $MgCl_2$: $[MgCl_2]_t = [ATP]_t + 2[ADP]_t$. Under these conditions fractional conversion of the nucleotides to the Mg–complex was relatively constant, and MgADP appeared to be a classical competitive inhibitor (Fig. 6). This effect, however, need not be due to product inhibition (implying the

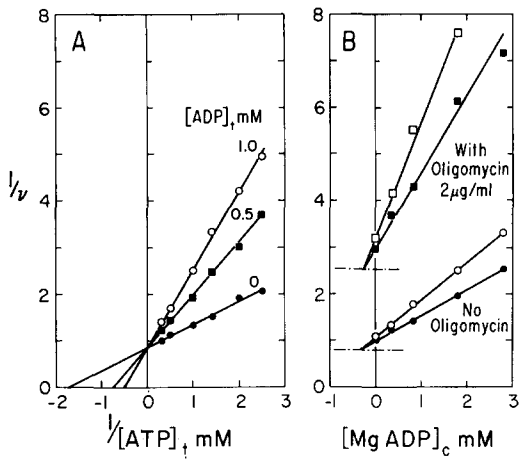

Fig. 6. Effects of ADP on the $(Na^+ + K^+)$-dependent ATPase. Experiments presented in Panel A were performed as in Fig. 1 with the concentrations of total ATP indicated, but with the total $MgCl_2$ concentration equal to the ATP concentration plus twice the ADP concentration, in the absence of ADP (●—●) or with 0.5 mM (■—■) or 1.0 mM ADP (○—○). In Panel B these results are plotted in the form of a Dixon plot in terms of the calculated MgADP concentration against the reciprocal of the velocity with 3 mM (●—●) and 2 mM (○—○) ATP, with a horizontal line at the reciprocal of the maximal velocity. Also presented in Panel B are results of identical experiments with MgADP and 3 mM (■—■) and 2 mM (□—□) ATP, but in the presence of 2 μg/ml oligomycin as well.

terminal release of ADP) but could result from dead-end inhibition, whereby a substance reacts with the enzyme to form a catalytically inert complex. Unfortunately, there is no straightforward method to distinguish in this system between product and dead-end inhibition.

In similar experiments varying the (calculated) MgADP level at essentially constant MgATP values gave a Dixon plot consistent with competitive inhibition, and a "K_i" of 0.3 mM (Fig. 6; Table I). Oligomycin had little effect on the "K_i" for MgADP (Fig. 6), in accord with its minimal effects on the K_m for MgATP (Fig. 1).

Effects of $CaCl_2$

A recent report [3] describes inhibition by $CaCl_2$ as being non-competitive

toward the substrate, although an earlier study [17] showed competition between MgATP and Ca, as the CaATP complex. Since it seemed peculiar that Mg^{2+} would be unable to counteract inhibition due to Ca^{2+}, the implication of non-competitive inhibition, this issue was reinvestigated.

$CaCl_2$ was a potent inhibitor of the ATPase (Fig. 7), but, as originally stressed

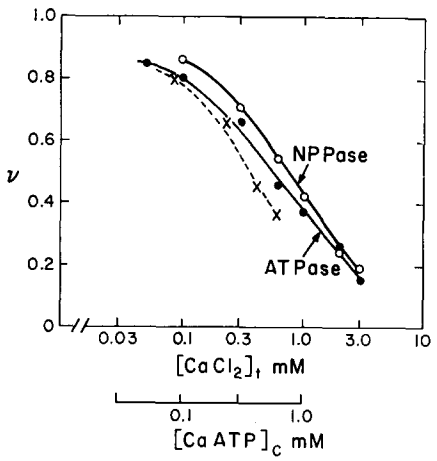

Fig. 7. Effects of Ca^{2+} on the ($Na^+ + K^+$)-dependent ATPase and K^+-dependent nitrophenylphosphatase. The enzyme preparation was incubated in the standard media for measuring ATPase (●—●) and nitrophenylphosphatase, NPPase (○—○), activities (see Methods and Materials) in the presence of the $CaCl_2$ concentrations indicated. In both cases velocity is expressed relative to that in the standard medium without $CaCl_2$. In addition, ATPase activity is also plotted (×---×) as a function of the calculated CaATP concentration in the presence of 3 mM $MgCl_2$, 3 mM ATP, and the added $CaCl_2$.

by Epstein and Whittam [17], investigations must consider Ca–ATP binding, which is nearly as strong as Mg–ATP binding [12]. Consequently, a simple plot of $CaCl_2$ concentration against activity at a given $MgCl_2$ and ATP level is misleading, because Ca^{2+}, Mg^{2+}, ATP, CaATP, and MgATP concentrations are highly interdependent. Similarly, adding a constant concentration of $CaCl_2$ while varying $MgCl_2$ and ATP at a constant 1:1 molar ratio results not only in markedly changing CaATP concentrations, but also accentuates changes in the extent of Mg–ATP complexing.

This difficulty can be minimized by two alternate approaches. The concentrations of both ATP and $MgCl_2$ can be varied at constant total $CaCl_2$ concentrations, but with $[ATP]_t = [MgCl_2]_t + [CaCl_2]_t$ (Fig. 8A): the concentration of MgATP varies only from 94–89% of the added $MgCl_2$ over the range studied, although the CaATP concentration deviates somewhat more, from 87–78% of the added $CaCl_2$. Better control is obtained when the $MgCl_2$ concentration is varied with a constant 3 mM ATP and 0.3 mM $CaCl_2$ (Fig. 8B). Here, with $MgCl_2$ concentrations from 2 mM downward the CaATP represents 96–99% of the added $CaCl_2$, and the MgATP represents from 98–100% of the added $MgCl_2$. In this latter approach there remains, however, the problem of inhibition by free ATP.

In both these cases where CaATP and MgATP levels are controlled (Fig. 8) it is clear that CaATP was the inhibitory species, rather than free Ca^{2+}. Moreover, the

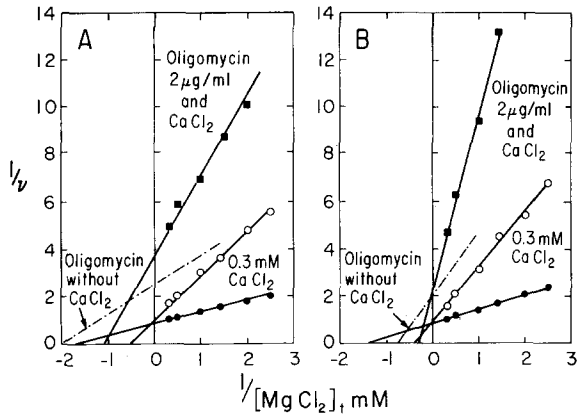

Fig. 8. Effects of Ca^{2+} on the (Na^+ + K^+)-dependent ATPase. Panel A shows experiments that were performed as in Fig. 1 with both $MgCl_2$ and ATP varied together, except that the total concentration of ATP was kept equal to the total concentration of $MgCl_2$ plus that of added $CaCl_2$, when present. Experiments presented are in the absence of $CaCl_2$ (●—●), with 0.3 mM $CaCl_2$ (○—○), and with 0.3 mM $CaCl_2$ plus 2 μg/ml oligomycin (■—■). In addition, a dashed line representing the relationship in the presence of oligomycin but absence of $CaCl_2$, taken from Fig. 1, is included. In Panel B are presented similar experiments, but with the total ATP concentration constant at 3 mM and with $MgCl_2$ varied in the absence (●—●) or presence (○—○) of 0.3 mM $CaCl_2$, or with 0.3 mM $CaCl_2$ plus 2 μg/ml oligomycin (■—■). Again, the dashed line represents the relationship in the presence of oligomycin but the absence of $CaCl_2$, taken from Fig. 2.

form of inhibition seen in double reciprocal plots was "mixed", with a strong implication of competitive interactions, as originally proposed by Epstein and Whittam [16] and as seems plausible.

The apparent failure to overcome totally the inhibition at infinite substrate concentration (as manifested graphically by intersections to the left of the ordinate) could be due simply to additional inhibitory actions of Ca^{2+}. Since it is known that certain divalent cations decrease the apparent affinity for monovalent cations [6, 10], the effect of Ca^{2+} on Na^+-activation was measured (Fig. 9): 0.3 mM $CaCl_2$ in-

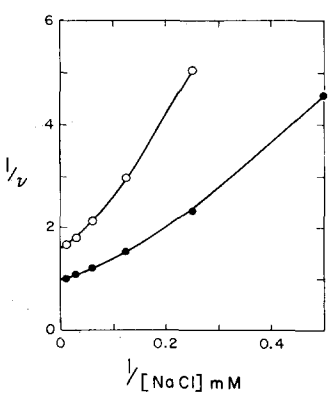

Fig. 9. Effects of Ca^{2+} on the response of the (Na^+ + K^+)-dependent ATPase to NaCl. The enzyme preparation was incubated in the standard medium but with the concentration of NaCl indicated, in the absence (●—●) or presence (○—○) of 0.3 mM $CaCl_2$.

creased the $K_{0.5}$ (the concentration for half-maximal activation) from 5.2 to 7.2 mM.

If the deviation from the pattern of classical competitive inhibition is due to independent actions such as this, the competition between CaATP and MgATP may then be approximated by the standard formulae [15], and a "K_i" for CaATP of about 0.12 mM can be calculated from Fig. 8A and Fig. 8B (Table III).

TABLE III

KINETIC PARAMETERS FOR INHIBITION BY CALCIUM

Values are taken from Fig. 8, with calculations by standard formulae [15].

Conditions	Apparent kinetic parameter (mM)		
	"K_m" for MgATP		"K_i" for CaATP
	without $CaCl_2$	with $CaCl_2$	
[ATP] = [$MgCl_2$] + [$CaCl_2$]			
$MgCl_2$ varied with 0.3 mM $CaCl_2$	0.59	1.9	0.11
plus oligomycin (2 μg/ml)	0.50	0.96	0.26
3 mM ATP and 0.3 mM $CaCl_2$			
$MgCl_2$ varied	0.79	2.6	0.13
plus oligomycin (2 μg/ml)	1.4	3.3	0.22

Oligomycin influenced the inhibition by Ca^{2+} as well (Fig. 8), and in the presence of 2 μg/ml oligomycin the calculated "K_i" for CaATP was increased (Table III). However, interpretation of the effects of oligomycin is uncertain here because of the interdependent variables; for example, in both Fig. 8A and Fig. 8B the concentration of free Mg^{2+} decreases with total $MgCl_2$, which could account for the "competitive" aspects of the inhibition by oligomycin (cf. Fig. 2 and Table II). In any case, it is clear that there was no synergism between the inhibition by Ca^{2+} and by oligomycin.

K^+-dependent nitrophenylphosphatase activity

The (Na^+ + K^+)-dependent ATPase exhibits K^+-dependent phosphatase activity that apparently reflects the terminal hydrolytic processes of the overall ATPase reactions [18]. This activity may be examined with nitrophenylphosphate as substrate, and since the dissociation constant for Mg–nitrophenyl phosphate is much higher [10] than that for Mg–ATP these experiments permit another approach to substrate–cation–enzyme interactions.

With 3 mM nitrophenylphosphate and 3 mM $MgCl_2$ only 27% of either is in the form of the complex. As the $MgCl_2$ is lowered the percentage of the total Mg^{2+} existing as the complex increases only slightly, reaching 32% of 0.5 mM total $MgCl_2$. Over this range of concentrations phosphatase activity appeared to follow Michaelis–Menten kinetics, with a "K_m" in terms of total $MgCl_2$ of 0.87 mM (Fig. 10A). If these experiments represented velocity as a function of the Mg–nitrophenyl phosphate complex, then the "K_m" would be 0.32 mM, lower than for the MgATP complex (and ignoring possible inhibition by free nitrophenylphosphate).

Ca^{2+} strongly inhibited the phosphatase activity (Fig. 7), although in terms of

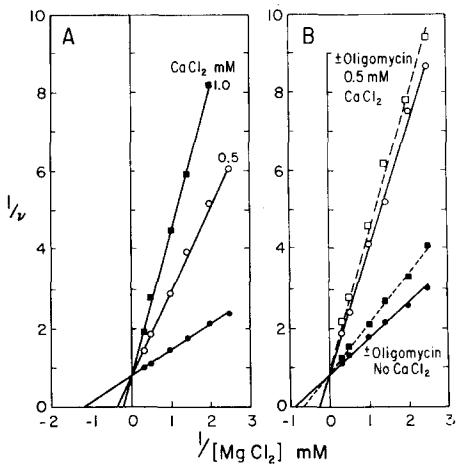

Fig. 10. Effects of substrate and divalent cations on the K^+-dependent nitrophenylphosphatase. Panel A shows experiments in which the enzyme preparation was incubated in the standard medium but with the concentration of $MgCl_2$ shown, in the absence of $CaCl_2$ (●—●), or with 0.5 mM (○—○) or 1.0 mM (■—■) $CaCl_2$. In Panel B are presented similar experiments in which 10 mM NaCl was included in all cases in the absence of $CaCl_2$ (●—●) alone or with 10 μg/ml oligomycin (■—■), or in the presence of 0.5 mM $CaCl_2$ (○—○) alone or with 10 μg/ml oligomycin (□---□).

total $CaCl_2$ added the inhibition was slightly less than against the ATPase (which may represent the difference in efficacy or affinity of Ca^{2+} versus CaATP). Toward $MgCl_2$, $CaCl_2$ appeared to be a classical competitor (Fig. 10A) with a "K_i" of 0.2 mM, appreciably larger than the calculated "K_i" for CaATP against MgATP. $EuCl_3$ was also a competitive inhibitor toward $MgCl_2$, but with a "K_i" of 0.02 mM, and when $MnCl_2$ was substituted for $MgCl_2$, $CaCl_2$ was still a competitive inhibitor with a "K_i" of 0.2 mM (data not presented).

To assess the effects of oligomycin it is necessary to include NaCl in the phosphatase incubation medium [8]. In the presence of NaCl and oligomycin the "K_m" for $MgCl_2$ was increased to 1.5 mM (Fig. 10B, Table IV); the "K_i" for $CaCl_2$ was also increased, to 0.38 mM.

TABLE IV

KINETIC PARAMETERS FOR THE K^+-DEPENDENT NITROPHENYLPHOSPHATASE

Values are taken from Fig. 10, with calculations by standard formulae [15].

Conditions	Apparent kinetic parameter (mM)	
	"K_m" for $MgCl_2$	"K_i" for $CaCl_2$
$MgCl_2$ varied with 3 mM nitrophenylphosphate	0.87	
plus 0.5 mM $CaCl_2$	2.8	0.22
plus 1.0 mM $CaCl_2$	5.0	0.21
$MgCl_2$ varied with 3 mM nitrophenylphosphate and 10 mM NaCl	1.2	
plus oligomycin 10 μg/ml	1.5	
plus 0.5 mM $CaCl_2$	3.7	0.23
plus oligomycin and $CaCl_2$	3.6	0.38

$MgCl_2$ and inactivation by Be^{2+}

To examine the binding of Mg^{2+} to the enzyme the technique using ATPase inactivation by Be^{2+} has the advantage of permitting measurements in the absence of substrate [11, 19]. Thus problems of ATP (and nitrophenylphosphate) complexing are avoided. In this approach the rate of inactivation is measured as a function of Mg^{2+} concentration by means of initial incubations (with $BeCl_2$, KCl, and various $MgCl_2$ concentrations) during which inactivation occurs, followed by brief assay incubations to measure the remaining activity. Plots of the reciprocals of the pseudo first-order rate constants for inactivation, k_{in}, against the reciprocals of the $MgCl_2$ concentrations are kinetically equivalent to Lineweaver–Burk plots, and allow calculation of the dis-

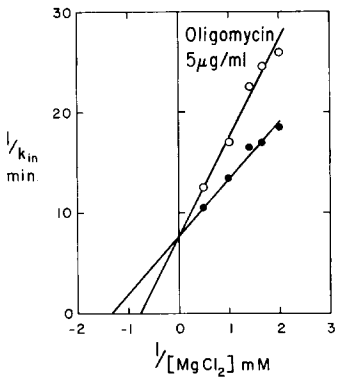

Fig. 11. Effects of Mg^{2+} and oligomycin on the inactivation of the $(Na^+ + K^+)$-dependent ATPase by $BeCl_2$. The enzyme preparation was first incubated with 50 mM Tris–HCl (pH 7.8), 50 μM $BeCl_2$, 10 mM KCl, and the concentration of $MgCl_2$ indicated, and the remaining activity then measured by adding 4 vol. of incubation medium (see Methods and Materials). The pseudo first-order rate constant for inactivation is plotted against the $MgCl_2$ concentration in double reciprocal form, for experiments in which the initial inactivating incubation was performed in the absence (●—●) or presence (○—○) of 5 μg/ml oligomycin.

sociation constant, K_D, from the intersection with the abscissa [11, 19]. In the presence of 10 mM KCl (both Mg^{2+} and K^+ are necessary for inactivation) the K_D for $MgCl_2$ was 0.77 mM (Fig. 11). In the presence of oligomycin the K_D was increased to 1.33 mM.

DISCUSSION

In vivo the MgATP concentration in rat brain is about 2 μmoles/g wet weight, with a free Mg^{2+} concentration about 0.6–1 μmole/g wet weight [13]; correspondingly, the concentrations usually examined in studies on the $(Na^+ + K^+)$-dependent ATPase in vitro are near these values. Under such conditions MgATP is clearly the substrate. In questioning this Hegyvary and Post [2] presumably were enquiring about the nature of the site(s) receiving MgATP: whether Mg^{2+} and ATP could bind independently. The experiments here show that although separate binding is indicated for free Mg^{2+} and ATP the affinity for MgATP is considerably higher (as reflected in the "K_m" for MgATP, representing an upper limit to the actual dissociation constant).

Although free Mg^{2+} was not a competitive inhibitor to MgATP, free ATP was, suggesting that while free ATP could diminish MgATP binding (by ATP at its own site occluding the Mg^{2+}-site?), bound Mg^{2+} at the active site did not hinder MgATP binding (by simple exchange with the Mg of the nucleotide?). These considerations are represented diagrammatically in Fig. 12.

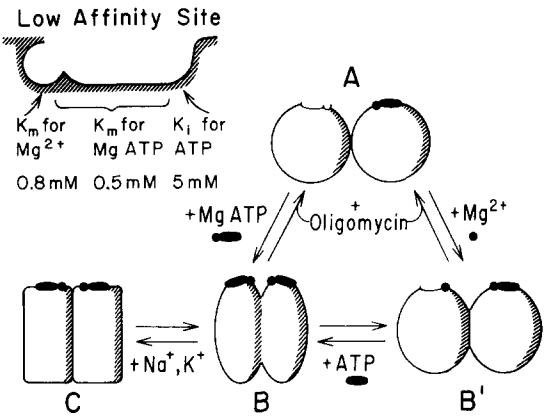

Fig. 12. Diagrammatic representation of the interaction of Mg^{2+} and ATP with the enzyme depicted as a two-subunit complex. Form A represents occupancy of the high-affinity substrate site, and Form B occupancy of the low-affinity site as well. In addition, the ability to exhibit cooperative homotropic responses to Na^+ and K^+, which is minimized by oligomycin [8] and low divalent cation concentrations [6], is represented by Form C.

By contrast, Nørby and Jensen [19] and Hegyvary and Post [2] demonstrated binding of free ATP to the enzyme, with a dissociation constant three orders of magnitude lower than the "K_m" seen here for MgATP. An explanation offered by Hegyvary and Post that this disparity represented a K^+-induced diminution in affinity seems unlikely since Na^+, in the concentration ratio to K^+ used for measuring "K_m", overcomes the K^+-induced changes (Fig. 4 of ref. 2). Because the binding experiments measure a dissociation constant, K_D, and

$$K_m = \frac{(k_{-1}) + (k_{+2})}{(k_{+1})} = K_D + \frac{k_{+2}}{k_{+1}}$$

the measured K_m might still be reconciled with K_D if k_{+2} were three orders of magnitude greater than k_{-1}. An alternative explanation employs the observations [20, 21] that there are at least two distinguishable K_m values for MgATP on the enzyme: a high affinity site with a K_m of $0.2\text{–}3 \cdot 10^{-6}$ M, and a low affinity site in the 10^{-4} M range. Thus measurements of ATP binding would seem to refer to the first class of sites (albeit with ATP rather than MgATP), whereas routine studies on K_m refer to the second set. Assuming that both sets are present on the same enzyme (although probably on different subunits), then the ATPase as usually examined in vitro, and as it functions in vivo, would be saturated at the high affinity sites.

Recently Levitzki et al. [23] proposed that for a significant class of enzymes only half the active sites are operative at a given time, and Stein et al. [24] suggested

that the ATPase was such an enzyme. Considerations of nucleotide binding would fit such a scheme, where ATP is hydrolyzed at only one site, hydrolysis either alternating between the two or occurring at one while the other serves a regulatory function, as E–ATP or E–P. This formulation, with ATP interacting at only the high affinity sites (Form A, Fig. 12), or with the high affinity sites saturated and ATP interacting with the low affinity sites as well (Form B, Fig. 12), may pertain to a number of issues: alternative catalytic characteristics (e.g. hydrolysis versus exchange), response to modifiers (e.g. oligomycin), and binding of inhibitors (e.g. ouabain). Moreover, this formulation could explain the puzzling observation that low concentrations of nucleotides stimulate phosphatase activity [10, 25, 26]. The stimulation apparently results from phosphorylation of the enzyme [11, 25, 27], and thus it would seem unlikely that for a single catalytic site occupancy by this enzyme–phosophate complex, which itself would be subject to hydrolysis, could at the same time accelerate hydrolysis of nitrophenylphosphate.

With regard to alternative catalytic activities, in particular $(Na^+ + K^+)$-dependent ATP hydrolysis versus Na^+-dependent ADP/ATP exchange, the conventional reaction scheme [4, 5]

$$E_1 \underset{\longleftarrow}{\xrightarrow{+MgATP}} E_1\text{–}P \xrightarrow{+Mg^{2+}} E_2\text{–}P \xrightarrow{-Mg^{2+}} E_1 + P_i \tag{1}$$

depicts a cyclical conversion of E_1 to E_2 to E_1, with a cyclical binding and release of free Mg^{2+}. An alternative scheme [6, 8]

$$\begin{array}{ccccc} E_I & \underset{\longleftarrow}{\xrightarrow{+MgATP}} & E_I\text{–}P & \rightarrow\!\!\rightarrow & E_I + P_i \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ E_{II} & \underset{\longleftarrow}{\xrightarrow{+MgATP}} & E_{II}\text{–}P & \rightarrow\!\!\rightarrow & E_{II} + P_i \end{array} \tag{2}$$

proposes that the two types represent different states of the enzyme, dependent on Mg^{2+} and ATP levels, without a requirement for cyclical conversion: at low Mg^{2+} and MgATP levels E_I predominates with prominent exchange activity; at high Mg^{2+} and MgATP levels E_{II} predominates with prominent hydrolytic activity. In Scheme 1 oligomycin blocks conversion of E_1–P to E_2–P; in Scheme 2 it favors the E_I state, with its enzymatic characteristics, over the E_{II} state.

The distinguishing feature of Scheme 1 is the inclusion of a Mg^{2+}-site, distinct from the substrate site, that must bind Mg^{2+} to convert E_1–P to E_2–P, and that must also discharge its bound Mg^{2+} with each enzymatic cycle to form E_1 again. Certain characteristics of such a site may be deduced from the available data. As free Mg^{2+} is decreased conversion of E_1–P to E_2–P would be slowed, and an upper limit for the K_m of the Mg^{2+}-binding site may be estimated. If the sole effect of reducing $MgCl_2$ with 3 mM ATP was to lower free Mg^{2+}, then the concentration of free Mg^{2+} at half-maximal velocity would represent the K_m for this site: 5 μM free Mg^{2+} when $MgCl_2$ is 0.8 mM (Fig. 2). It is clear that reducing Mg^{2+} slows the reaction by other means (e.g. reducing MgATP levels), but this gives an upper limit for K_m. An affinity of enzyme for Mg^{2+} in the micromolar range is not implausible, although it represents a higher affinity for Mg^{2+} than ATP has.

On the other hand, in order to form E_1 again in Scheme 1 this bound Mg^{2+}

must be discharged, meaning that the affinity must be drastically reduced. If the sole effect of excess free Mg^{2+} was to slow the ATPase by diminishing the obligatory dissociation of E–Mg, then the concentration for half-maximal velocity should give the affinity at this point in the cycle. A "K_i" of about 40 mM was found for inhibition by free Mg^{2+} (Fig. 5); this is a lower limit for the true K_i at such a site since excess Mg^{2+} undoubtedly inhibits by other mechanisms, e.g. competition for Na^+-sites. Thus a model incorporating cyclical addition and release of Mg^{2+} (Scheme 1) implies a cyclical change in affinity for Mg^{2+} of at least four orders of magnitude, which strains plausibility.

Scheme 2, however, does not require that the regulatory Mg^{2+} be added and lost with each reaction cycle, and the enzyme can continue in the E_{II} state. In this formulation the regulatory Mg^{2+} might be at distinct control sites, or at the low affinity MgATP site either with ATP (Form B of Fig. 12) or alone (Form B'); if there are more than two subunits then considerable latitude exists for combinations of Mg^{2+} and MgATP binding. Form A of the allosteric complex [6] thus reflects occupancy of only the high affinity site, with catalytic characteristics of the E_I (E_1–P) state: good ADP/ATP exchange and poor hydrolysis (although hydrolysis with a low K_m), whereas Form B reflects occupancy of the low affinity site as well, with good hydrolysis (and a high K_m) but poor ADP/ATP exchange. Oligomycin would favor the E_I state (Form A or a close structural analog), reducing the affinity for Mg^{2+} (Fig. 11) while Mg^{2+} antagonizes oligomycin inhibition [8]. MgATP should also antagonize oligomycin inhibition, but the issue is complicated by oligomycin favoring a form with a higher affinity for MgATP; consequently, at micromolar concentrations of MgATP oligomycin inhibits less than at millimolar concentrations [8]. This would tend to produce a picture of increasing inhibition at high MgATP concentrations, whereas antagonism should produce decreasing inhibition. In the range of MgATP concentrations examined here an intermediate case, essentially constant inhibition was seen (Fig. 1), which may be consistent with the model although it cannot be construed as support for it.

Recently Tobin et al. [3] proposed that Ca^{2+} was another agent for producing the E_1–P form of the enzyme: in the presence of Ca^{2+} phosphorylation of the enzyme was slowed somewhat, ADP/ATP exchange was greater than with equimolar Mg^{2+}, and ATP hydrolysis was markedly reduced. In the formulations here it would seem that divalent cations serve a dual role: to participate with nucleotide in the catalytic events forming the E–P complex, and to interact with the enzyme to favor the E_{II} state (Form B or Form B'). In contrast to MgATP, CaATP apparently serves as a mediocre substrate for forming E–P (consequently acting as a competitor to MgATP) and an almost ineffective allosteric effector, in the latter role failing to produce E_{II}. CaATP thus appears to allow the E_I state to exist by default, rather than by selection (cf. oligomycin).

ACKNOWLEDGEMENTS

I wish to thank Mr Jonathan Mallov and Miss Grace Marin for thoughtful technical assistance, and Mr Edward Matyas for the computer programming. This work was supported by U.S. Public Health Service research grant NS-05430 and Biotechnology Resources grant RR-00353.

REFERENCES

1 Skou, J. C. (1965) Physiol. Rev. 45, 596–617
2 Hegyvary, C. and Post, R. L. (1971) J. Biol. Chem. 246, 5234–5240
3 Tobin, T., Akera, T., Baskin, S. I. and Brody, T. M. (1973) Mol. Pharmacol. 9, 336–349
4 Fahn, S., Koval, G. J. and Albers, R. W. (1966) J. Biol. Chem. 241, 1882–1889
5 Post, R. L., Kume, S., Tobin, T., Orcutt, B. and Sen, A. K. (1969) J. Gen. Physiol. 54, 306S–326S
6 Robinson, J. D. (1972) Biochim. Biophys. Acta 266, 97–102
7 Inturrisi, C. E. and Titus, E. (1968) Mol. Pharmacol. 4, 591–599
8 Robinson, J. D. (1971) Mol. Pharmacol. 7, 238–246
9 Robinson, J. D. (1967) Biochemistry 6, 3250–3258
10 Robinson, J. D. (1969) Biochemistry 8, 3348–3355
11 Robinson, J. D. (1973) Arch. Biochem. Biophys. 156, 232–243
12 O'Sullivan, W. J. and Perrin, D. D. (1964) Biochemistry 3, 18–26
13 Veloso, D., Guynn, R. W. Oskarsson, M. and Veech, R. L. (1973) J. Biol. Chem. 248, 4811–4819
14 Hexum, T., Samson, Jr, F. E. and Himes, R. H. (1970) Biochim. Biophys. Acta 212, 322–331
15 Dixon, M. and Webb, E. C. (1964) Enzymes 2nd edn, pp. 316–331, Academic Press, New York
16 Schoner, W., Kramer, R., and Seubert, W. (1966) Biochem. Biophys. Res. Commun. 23, 403–408
17 Epstein, F. H. and Whittam, R. (1966) Biochem. J. 99, 232–238
18 Schoner, W. (1971) Angew. Chem. (Int. edn) 10, 882–889
19 Robinson, J. D. (1973) Biochim. Biophys. Acta 321, 662–670
20 Nørby, J. G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116
21 Neufeld, A. H. and Levy, H. M. (1969) J. Biol. Chem. 244, 6493–6497
22 Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. Tokyo 67, 693–711
23 Levitzki, A., Stallcup, W. B. and Koshland, Jr, D. E. (1971) Biochemistry 10, 3371–3378
24 Stein, W. D., Lieb, W. R., Karlish, S. J. D. and Eilam, Y. (1973) Proc. Natl. Acad. Sci. U.S. 70, 275–278
25 Rega, A. F., Garrahan, P. J. and Pouchan, M. I. (1968) Biochim. Biophys. Acta 150, 742–744
26 Yoshida, H., Nagai, K., Ohashi, T. and Nakagawa, Y. (1969) Biochim. Biophys. Acta 171, 178–185
27 Tobin, T., Baskin, S. I., Akera, T. and Brody, T. M. (1972) 8, 256–263