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THE $(Na^+ + K^+)$-DEPENDENT ATPase

MODE OF INHIBITION OF ADP/ATP EXCHANGE ACTIVITY BY $MgCl_2$

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SUMMARY

Na^+-dependent ADP/ATP exchange activity, of a $(Na^+ + K^+)$-dependent ATPase preparation from eel electric organ, was measured in terms of the incorporation of ^{14}C into ATP during incubations with unlabeled ATP and $[^{14}C]$ADP. Estimates of initial rates of exchange were possible by keeping changes in nucleotide concentrations, from both exchange and extraneous hydrolytic processes, to less than 10 %. Under these conditions, increases in $MgCl_2$ concentration, from 0.2 to 3 mM, generally inhibited this exchange activity. The concentrations of free Mg^{2+}, Mg · ATP, and Mg · ADP present, with a range of $MgCl_2$, ATP, and ADP concentrations, were calculated from measured dissociation constants. Inhibition was associated with Mg · ATP as well as with Mg^{2+}, at concentrations from 0.4 to 1 mM (Mg · ADP, in the same concentration range, probably inhibited also). The affinity of the enzyme for these inhibitors is in fair correspondence with demonstrated affinities for Mg^{2+}, Mg · ATP, and Mg · ADP at low affinity substrate sites, measured kinetically. These observations are considered in terms of a dimeric enzyme with high and low affinity substrates sites: ADP/ATP exchange being catalyzed at the high affinity sites, with inhibition occurring through occupancy by Mg^{2+}, Mg · ATP, or Mg · ADP, of the low affinity sites, thereby pulling the reaction process away from those steps involved in exchange.

INTRODUCTION

Formulations of the reaction sequence of the $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) have been greatly influenced by early observations on the Na^+-dependent ADP/ATP exchange reaction that is also catalyzed by this enzyme. Albers and associates [1, 2] found that concentrations of $MgCl_2$ that were optimal for the overall ATPase reaction markedly inhibited the ADP/ATP exchange reaction. To account for this inhibition, they proposed that the enzyme was first phosphorylated by ATP to form E_1-*P*, which participated in the exchange reaction: subsequent binding of Mg^{2+} to E_1-*P* formed a lower energy phosphoenzyme,

E_2-*P*, that could not react with ADP but was susceptible to K^+-dependent hydrolysis. From considerations of symmetry, E_2 must return to E_1 with a release of Mg^{2+}.

$$E_1 \xrightarrow[\mathrm{Mg^{2+}, Na^+}]{\mathrm{ATP}} E_1\text{-}P \xrightarrow[\mathrm{Mg^{2+}}]{} E_2\text{-}P \xrightarrow[\mathrm{K^+}]{} E_1 + P_i + Mg^{2+}$$

Support for this scheme was provided by Post et al. [3], who showed that after formation of a phosphoenzyme in the presence of $MgCl_2$, the release of P_i on addition of K^+ was strongly inhibited by EDTA. These data thus fit a process in which chelation of Mg^{2+} by EDTA converts the phosphoenzyme from a form sensitive to K^+, E_2-*P*, to a form insensitive to K^+ but sensitive to ADP, E_1-*P*.

The proposed scheme satisfies these observations convincingly, although other considerations raised possibilities of alternative formulations [4, 5]. More recently, however, two lines of evidence cast doubts on the original interpretation. (i) Measurements of ATP hydrolysis in the presence of a wide range of Mg^{2+}, Mg · ATP, and ATP concentrations showed that if free Mg^{2+} must bind to and be released from the enzyme with each hydrolytic cycle, then the cyclical change in affinity for Mg^{2+} must be at least 60 000-fold [6]. (ii) The inhibition by EDTA of the K^+-induced hydrolysis of the phosphoenzyme was demonstrated not to result from chelation of Mg^{2+} [7].

If, as these observations suggest, the reaction sequence of the ATPase does not proceed through a Mg^{2+}-dependent conversion of E_1-*P* to E_2-*P*, then the original experiments on the exchange reaction are unexplained. The study described here was undertaken to re-examine the effects of $MgCl_2$ on the Na^+-dependent ADP/ATP exchange. For this purpose, the ATPase preparation from eel electric organ was chosen, not only because it is the preparation used in the original experiments, but also because it is free of contaminating adenylate kinase activity.

In confirmation of the original experiments, $MgCl_2$ was found to inhibit ADP/ATP exchange. However, when this inhibition is examined in terms of the calculated concentrations of Mg^{2+}, Mg · ATP, and Mg · ADP, no simple correlation with free Mg^{2+} is apparent. Instead, inhibition is associated with certain concentrations of either Mg^{2+}, Mg · ATP, or Mg · ADP. These findings are considered in terms of an enzyme with two classes of substrate sites [8]. Catalysis of the exchange reaction would then be at the high affinity substrate sites (those seen in binding studies [9, 10] and in kinetic studies with μM substrate concentrations [8, 11]). On the other hand, inhibition would result from occupancy by Mg^{2+}, Mg · ATP, or Mg · ADP of the low affinity sites (those seen in the usual kinetic determinations of K_m), sites involved in the overall ATPase reaction sequence [8].

METHODS AND MATERIALS

The ATPase preparation from electric organ of the eel (*Electrophorus electricus*) was isolated according to Albers et al. [12].

ADP/ATP exchange activity was measured in terms of the incorporation of ^{14}C into ATP during incubations at 30 °C with unlabeled ATP and [U-^{14}C]ADP (about $5 \cdot 10^5$ cpm). Incubation media contained 30 mM histidine · HCl/Tris (pH 7.8), the concentrations of ADP, ATP (both as Tris salts), and $MgCl_2$ indicated, plus either 20 mM NaCl or 0.1 mM ouabain. Final volume was 50 μl. The reaction was terminated by placing the incubation tubes in a boiling water bath for 2 min [1]. To separate ATP, ADP, and AMP, a 20 μl portion of the reaction mixture was spotted on washed polyethyleneimine-impregnated cellulose-coated plastic films (Brinkmann Instrument Co.) for thin-layer chromatography. Unlabeled carrier ATP, ADP, and AMP were added to the mixture before spotting. After development at room temperature in 1.2 M LiCl, the nucleotides were located by absorption of ultraviolet light (R_F values for ATP, ADP, and AMP were, respectively, 0.20, 0.44, and 0.63), and these regions were cut from the films for liquid scintillation counting in Scintisol-Complete (Isolabs, Inc.). Controls indicated that efficiency of counting was independent of the area of radioactivity on the film, within the range encountered in these experiments. Recovery of added radioactivity was monitored by spotting an equal volume of the reaction mixture on polyethyleneimine-impregnated cellulose films after they had been soaked in 1.2 M LiCl. The rate of incorporation of ^{14}C into ATP measured in the presence of NaCl, less the rate in the presence of 0.1 mM ouabain, was considered to be the Na^+-dependent ADP/ATP exchange rate [1, 13].

As an indicator of adenylate kinase activity in these experiments, radioactivity on the polyethyleneimine-impregnated cellulose films in the region of AMP was sought.

Total ATPase activity was measured in terms of the production of [^{14}C]ADP in parallel incubations with [U-^{14}C]ATP and unlabeled ADP. The reaction was terminated, the nucleotides separated by thin-layer chromatography, and the radioactivity measured as described above. Alternatively, when exchange experiments involved 1 mM (or higher) concentrations of ATP, ATPase activity was measured in terms of P_i production, as previously described [14].

($Na^+ + K^+$)-dependent ATPase activity was measured at 30 °C in terms of P_i production [14]. The standard medium contained 30 mM histidine · HCl/Tris (pH 7.8), 3.5 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, and 10 mM KCl. Activity in the absence of added Na^+ and K^+ was measured concurrently, and this activity was subtracted from the total activity in the presence of Na^+ and K^+ to give the ($Na^+ + K^+$)-dependent activity [14]. K^+-dependent phosphatase activity was measured at 30 °C in terms of *p*-nitrophenol production during incubation with *p*-nitrophenyl phosphate, as previously described [15]. The incubation media contained 30 mM histidine · HCl/Tris (pH 7.8), the concentrations of $MgCl_2$ and *p*-nitrophenyl phosphate (as the Tris salt) indicated, and 10 mM KCl. Activity in the absence of added K^+ was measured concurrently, and was subtracted from the total activity in the presence of K^+ to give the K^+-dependent activity [15].

The dissociation constant for Mg · ADP was measured by method (a) of Watanabe et al. [16]. Fluorescence of the 8-hydroxyquinoline · magnesium complex was determined in media containing 30 mM histidine · HCl/Tris (pH 7.8), 20 mM NaCl, 0.5 mM 8-hydroxyquinoline, and a range of ADP and $MgCl_2$ concentrations.

Data presented are averages of five or more experiments, performed in duplicate.

[U-^{14}C]ADP (500–600 Ci/mol) and [U-^{14}C]ATP (500–600 Ci/mol) were purchased from Amersham/Searle. Unlabeled ATP, ADP, AMP, and *p*-nitrophenyl phosphate were purchased from Sigma. Electric eels were obtained from World Wide Scientific Animals, Ardsley, N.Y.

RESULTS AND DISCUSSION

Dissociation constant for Mg · ADP. In order to distinguish between effects due to free Mg^{2+}, free nucleotides, and Mg · nucleotide complexes, as concentrations of $MgCl_2$ and nucleotides are varied, it is obviously necessary to know the concentration of each. This, in turn, requires evaluation of the Mg · ADP and Mg · ATP dissociation constants under the experimental conditions used. The dissociation constant for Mg · ATP in this medium was estimated, previously [6], to be 32 μM. Using this same technique [16], the dissociation constant for Mg · ADP was found to be 360 μM.

Na^+-dependent ADP/ATP exchange activity. When the enzyme was incubated with 1 mM $MgCl_2$, 1 mM ATP, and 1 mM ADP labeled with ^{14}C, ^{14}C-labeled ATP was produced. The rate of labeling of ATP increased linearly with time over short incubation periods (6–12 min) during which only a few percent of the total radioactivity added was incorporated into ATP. Thus, it was possible to evaluate the initial rate of ADP/ATP exchange under these conditions: 60 nmol per min per mg protein at 30 °C. Adding 20 mM NaCl to the incubation medium increased the rate of labeling, to 130 nmol per min per mg protein (for ease of comparison with other incubation conditions this rate of Na^+-dependent exchange was assigned a relative value of 100). Following the arguments of Fahn et al. [1] and Stahl [13], the Na^+-dependent exchange will be considered as representing the activity of the (Na^++K^+)-dependent ATPase enzyme, with the Na^+-independent exchange representing an extraneous enzymatic activity. Hereafter, the ADP/ATP exchange activity described will represent this Na^+-dependent activity, defined operationally as the difference between incubations with 20 mM NaCl and those without added NaCl but with 0.1 mM ouabain.

ATPase activity. A second complexity in measuring the exchange activity is the presence of ATPase activity. The electric organ ATPase preparation, like most (Na^++K^+) dependent-ATPase preparations in general use, exhibits a low level of activity in the absence of added Na^+ and K^+ (about 10 % of the activity with optimal levels of Na^+ and K^+). This Mg^{2+}-ATPase activity is believed to represent contaminating enzymatic activity [17]. In addition to ATP hydrolysis from this source, the (Na^++K^+)-dependent ATPase itself will hydrolyze ATP in the presence of Na^+ but absence of K^+; this Na^+-dependent ATPase activity of the (Na^++K^+)-dependent ATPase enzyme is, however, far smaller than the activity with both Na^+ and K^+. For these experiments the essential aspect is the presence of ATPase activity, from all sources. Clearly, these ATPase can dilute the [^{14}C]ADP added originally, by hydrolyzing unlabeled ATP to unlabeled ADP, thus resulting in an apparently lower rate of exchange activity (this would occur most readily when the ratio of ATP to ADP is high). Also, the action of these ATPases can lead to an apparently lower rate of exchange activity through hydrolysis of the [^{14}C]ATP formed, back to [^{14}C]ADP (this would occur most readily when the ratio of ATP to ADP is low). Because exchange and hydrolytic activities are catalyzed by the (Na^++K^+)-dependent ATPase, this is a problem with any enzyme preparation.

One approach to resolving this difficulty is the addition to the incubation media of inhibitors of the ATPase reaction, most commonly oligomycin and *N*-ethylmaleimide [1, 2]. However, oligomycin does not block the (Na^++K^+)-dependent ATPase totally [4], nor the Mg^{2+}-ATPase at all; oligomycin may also directly stimulate the exchange reaction [1]. Similar problems of specificity occur with sulfhydryl reagents [2, 18]. To avoid possible effects of such inhibitors on the exchange reaction itself, an alternative approach was chosen: incubation times and enzyme concentrations were selected so that changes in ATP and ADP were limited to less 10 % of their initial concentrations. Thus, changes both in [^{14}C]ADP level, due to the exchange activity and to dilution by unlabeled ADP, and in newly formed [^{14}C]ATP, due to ATPase activity, were minimized. Total ATPase activities (Na^+ dependent and independent) were measured in parallel incubations in terms of [^{14}C]ADP production from [^{14}C] ATP, and also, at higher ATP concentrations, in terms of P_i production: with 1 mM ADP, ATP, and $MgCl_2$, and 20 mM NaCl, the total ATPase activity was about two-thirds of the total exchange activity.

Adenylate kinase activity. Adenylate kinase activity is present in many (Na^+ + K^+)-dependent ATPase preparations [18–20], and for studies of exchange activity corrections have been necessary. In accord with the report by Albers et al. [12], the eel ATPase preparation did not exhibit measurable levels of adenylate kinase activity under these experimental conditions.

Effects of $MgCl_2$ on exchange activity. As the concentration of $MgCl_2$ added to the incubation media was raised from 0.2 to 3 mM the rate of Na^+-dependent ADP/ATP exchange activity decreased (Table I), in general agreement with earlier studies [1, 2, 19]. When the change in velocity is compared with the changes in free Mg^{2+}, however, no simple correspondence is apparent.

With 5 mM ATP and 1 mM ADP, successive increases in $MgCl_2$ concentration that led to a drastic decrease in exchange activity (Table I) are associated with low levels of free Mg^{2+} but with sharply increasing concentrations of the Mg · ATP

TABLE I

EFFECTS OF $MgCl_2$ ON Na^+-DEPENDENT ADP/ATP EXCHANGE ACTIVITY

Na^+-dependent ADP/ATP exchange activity was determined, as described in the text, from incubations with 1 mM ADP and the concentrations of ATP and $MgCl_2$ listed. Initial velocities of the exchange reaction are shown relative to that from incubations with 1 mM ATP and $MgCl_2$ defined as 100, ±S.E. Concentrations of free Mg^{2+} and the Mg · ATP and Mg · ADP complexes were calculated from the measured dissociation constants.

Concentrations of additions (mM)		Calculated concentrations of reactants (mM)			Velocity of exchange reaction
ATP	$MgCl_2$	Mg^{2+}	Mg · ATP	Mg · ADP	
0.2	0.2	0.029	0.096	0.075	54 ± 4
0.2	1.0	0.336	0.182	0.482	57 ± 3
0.2	2.0	1.060	0.194	0.746	31 ± 5
0.2	3.0	1.958	0.197	0.845	20 ± 4
1.0	0.2	0.007	0.175	0.018	163 ± 14
1.0	1.0	0.085	0.725	0.190	100
1.0	2.0	0.487	0.938	0.575	41 ± 3
1.0	3.0	1.249	0.975	0.776	24 ± 4
5.0	0.2	0.002	0.194	0.004	141 ± 11
5.0	1.0	0.008	0.971	0.021	74 ± 7
5.0	2.0	0.020	1.927	0.053	16 ± 3
5.0	3.0	0.042	2.852	0.106	4 ± 2

complex. In this case, inhibition of Na^+-dependent exchange activity would seem to result from an action of the Mg · ATP complex.

On the other hand, with 0.2 mM ATP and 1 mM ADP, successive increases in $MgCl_2$ also inhibited exchange activity (from 1 to 3 mM $MgCl_2$) Here, however, inhibition occurred with $MgCl_2$ concentrations causing little further increase in Mg · ATP levels. Instead, inhibition is associated with increased levels of Mg · ADP and free Mg^{2+}. These observations also support the contention, above, that in the presence of 5 mM ATP the inhibition is due to Mg · ATP rather than to free Mg^{2+}: with 0.2 mM ATP, raising the $MgCl_2$ concentration from 0.2 to 1.0 mM increased free Mg^{2+} to 0.3 mM, far beyond the highest concentration achieved in the presence of 5 mM ATP (0.04 mM), yet no inhibition was apparent.

With 1 mM ATP and ADP, successive increases in $MgCl_2$ again inhibited exchange activity. Here, inhibition is associated with increases in free Mg^{2+} and in both Mg · nucleotide complexes, although the correspondence between changes in inhibition and changes in concentration of agent is somewhat better with Mg · ADP and free Mg^{2+} than with Mg · ATP.

An alternative, or additional, factor in all these cases might be the concentration of free nucleotides, or the ratio of Mg · nucleotide to free. For ATP, however, the inhibition seen with 0.2 or 5.0 mM ATP (as $MgCl_2$ is increased) does not correspond well with decreases in free ATP nor with the ratio of free to Mg · ATP (Table I). For ADP, although a correspondence between inhibition and a decline in free ADP does not appear in experiments with 5 mM ATP, fair correspondence obtains in the presence of 0.2 and 1.0 mM ATP. Nevertheless, even if changes in free ADP concentration cannot represent the sole mechanism for inhibition by $MgCl_2$, these experiments do not rule out the possibility that such factors contribute to the observed inhibition. In any case, the data are not compatible with the original formulation [1] in which $MgCl_2$ inhibited exchange activity simply through occupying a unique site for free Mg^{2+} that governed the conversion of one phosphorylated form of the enzyme (E_1-*P*) to another (E_2-*P*).

TABLE II

EFFECTS OF $MgCl_2$ ON Na^+-DEPENDENT ADP/ATP EXCHANGE ACTIVITY AT LOW NUCLEOTIDE CONCENTRATIONS

Na^+-dependent ADP/ATP exchange was measured and the results are presented, as in Table I, except that the concentration of ADP was 50 μM throughout, and the initial velocities of the exchange reaction are shown relative to that with 50 μM ATP and $MgCl_2$, defined as 100.

Concentrations of additions (μM)		Calculated concentrations of reactants (μM)			Velocity of exchange reaction
ATP	$MgCl_2$	Mg^{2+}	Mg · ATP	Mg · ADP	
10	100	84	7	9	64 ± 4
50	50	25	22	3	100
50	100	60	33	7	95 ± 8
50	200	145	41	14	81 ± 5
50	500	426	47	27	39 ± 4
50	1000	916	48	36	10 ± 2
250	100	16	82	2	128 ± 12

Experiments with low concentrations of ATP and ADP. In the preceding experiments, exchange activity appeared to be inhibited by concentrations of Mg · ATP, Mg · ADP, and/or free Mg^{2+} in the concentration range 0.4–1 mM (Table I). A further approach to determining the effect of free Mg^{2+} would thus involve studying exchange activity in the presence of ADP and ATP concentrations below this range (Table II). With 50 μM ADP and ATP, successive increases in $MgCl_2$ again inhibited exchange activity, and this inhibition corresponds better with the concentration of free Mg^{2+} than with the Mg · ATP complex. Moreover, the sensitivity to free Mg^{2+} is in fair agreement with the sensitivity to free Mg^{2+} seen with 1 mM ADP and either 0.2 or 1 mM ATP (Table I).

Although the inhibition also corresponds with increases in the Mg · ADP complex, the level of Mg · ADP at near-maximal inhibition (Table II) is far below its level at near-maximal inhibition found in the preceding experiments (Table I). The concentrations of either free ATP or ADP that are associated with inhibition here do not correspond with those seen in the preceding experiments with higher nucleotide concentrations. The ratio of free ADP to Mg · ADP that is associated with inhibition under these conditions is in fair agreement with that seen in the presence of 1.0 mM ATP (Table I), but not with 0.2 or 5.0 mM ATP. Nevertheless, the possibility remains that an additional contributing factor in inhibition with $MgCl_2$ may be a decreased ratio of free ADP to Mg · ADP.

Apparent affinity for Mg^{2+}. These findings suggest that inhibition of the exchange activity could be effected through sites for free Mg^{2+} occupied at concentrations in the range 0.4–1 mM. To demonstrate the presence of such Mg^{2+} sites on the enzyme, the K_m for $MgCl_2$ of the K^+-dependent phosphatase reaction was examined. The K^+-dependent phosphatase reaction is catalyzed by the $(Na^+ + K^+)$-dependent

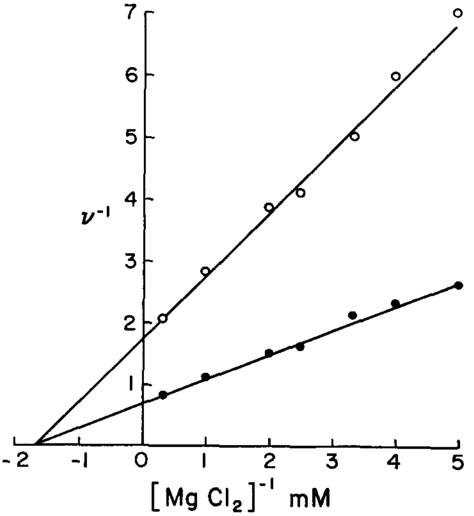

Fig. 1. Effect of $MgCl_2$ concentration of K^+-dependent phosphatase activity. Phosphatase activity was measured, as described under Methods and Materials, with the concentration of $MgCl_2$ indicated and with either 5 mM (●) or 1 mM (○) *p*-nitrophenyl phosphate as substrate. Data are presented in double-reciprocal form.

ATPase, and appears to reflect the terminal hydrolytic steps of the overall ATPase reaction [17]. With nitrophenyl phosphate as the substrate for the phosphatase reaction the apparent affinity for free Mg^{2+} can be estimated since the dissociation constant for the Mg · nitrophenyl phosphate complex is quite large [15]; previous studies [21] have indicated that these sites correspond to the Mg^{2+} aspect of the low affinity substrate sites for Mg · ATP. The K_m for $MgCl_2$ was 0.6 mM (Fig. 1), in accord with the apparent affinity for Mg^{2+} of a brain ATPase preparation [21] and with the concentration range at which free Mg^{2+} appears to inhibit the exchange reaction (Tables I and II). (Although earlier experiments [22] showed that K^+ acts as a competitor toward Mg^{2+} in the phosphatase reaction, the K_i for K^+, 70 mM, is large compared to the KCl concentration used here, 10 mM; thus the presence of KCl leads to only a slight overestimation of the true K_m.)

Apparent affinity for Mg · ADP and Mg · ATP. The apparent affinities for Mg · ATP and Mg · ADP at the low affinity substrates sites of the $(Na^+ + K^+)$-dependent ATPase [8] were estimated from substrate vs. velocity plots and from the effects of Mg · ADP as a competitor to Mg · ATP (Fig. 2). The K_m for Mg · ATP was 0.4 mM, and the K_i for Mg · ADP was 0.3 mM. Again, these apparent affinities correspond to the estimated affinities for the sites at which Mg · ATP and Mg · ADP seem to inhibit the exchange reaction. By contrast, the K_i for free ATP, as a competitor to Mg · ATP, was previously found to be 4 mM [6].

Estimating the dissociation constant for free ADP at the low affinity substrate sites is complicated by competition between ATP and ADP for Mg^{2+} (making

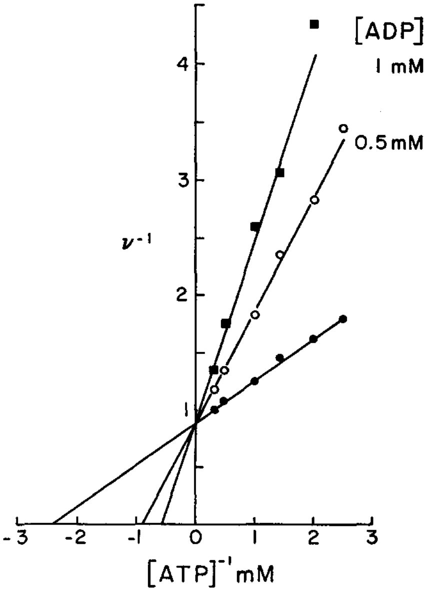

Fig. 2. Effects of ATP and ADP concentrations on $(Na^+ + K^+)$-dependent ATPase activity. ATPase activity was measured, as described under Methods and Materials, with the concentration of ATP indicated, without ADP (●), or with 1 mM (■) or 0.5 mM (○) ADP. In these experiments the $MgCl_2$ concentration was kept 1 mM greater than the sum of the ATP plus ADP concentration. Data are presented as in Fig. 1.

possible inhibition by free ATP as well). Nevertheless, because the affinity of ATP for Mg^{2+} is an order of magnitude greater than that of ADP, the K_i for ADP, measured with the $MgCl_2$ concentration held equal to the ATP concentration, can give a reasonable approximation. The K_i for ADP, so determined, was 5 mM (data not presented).

Apparent affinity for ADP in the exchange reaction. In the exchange reaction Mg · ATP must first phosphorylate the enzyme, an essential step for the overall ATPase reaction as well. Since Mg · ATP ostensibly inhibited exchange with an apparent affinity corresponding to that as a substrate for the overall ATPase, a seeming contradiction results. This dual role for Mg · ATP, as both substrate and inhibitor, is difficult to reconcile with a single class of substrate sites. The dilemma may be resolved by invoking two classes of nucleotide sites, one at which catalysis of the exchange reaction occurs, and one through which Mg · ATP or Mg · ADP or Mg^{2+} inhibits. Evidence for two classes of substrate sites includes measurements of dissociation constants for ATP on the order of 0.1 μM [9, 10] and 0.1 mM [8, 23], and Lineweaver-Burk plots of the substrate vs. velocity relationship of the $(Na^+ + K^+)$-dependent ATPase activity that, when extended to low Mg · ATP concentrations, are biphasic and in accord with the dissociation constants [8, 11].

If catalysis of the exchange reaction occurs at such high affinity substrate sites, while inhibition occurs through occupancy of the low affinity sites, then the K_m for the exchange reaction should coincide with that for the high affinity sites. Unfortunately, attempts to measure the K_m for the exchange reaction are beset with difficulties. Mg · ATP is required for phosphorylation of the enzyme, with free ATP, free ADP,

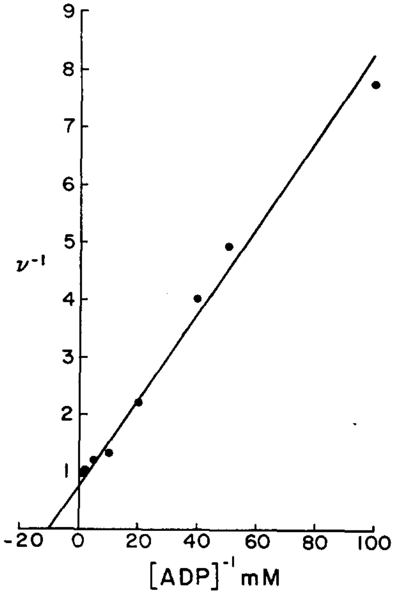

Fig. 3. Effect of ADP and ATP concentration on Na^+-dependent ADP/ATP exchange activity. ADP/ATP exchange activity was measured, as described under Methods and Materials, with the concentration of ADP indicated, and with an equimolar concentration of ATP. The $MgCl_2$ concentration was 0.2 mM throughout. Data are presented as in Fig. 1.

and Mg · ADP competitors at this step. [^{14}C]ADP, by which exchange activity is measured, must bind after unlabeled ADP (from the phosphorylating ATP molecule) has dissociated; it seems probable that ATP competes here with [^{14}C]ADP. The optimal ratio of ATP to ADP must reflect these two sets of competitions, and seems to lie near 1 : 1 (Table I). When ATP and ADP were varied at a 1 : 1 ratio an apparent K_m for ADP near 0.1 mM was found (Fig. 3). This value is lower than that for either Mg · ADP or ADP at the low affinity substrate sites. However, it is markedly higher than the apparent affinity for ADP at the high affinity sites, measured either directly in binding experiments, 0.3 μM [24], or as a competitor to ATP in kinetic experiments, 8 μM [8]. Part of this discrepancy might lie in the continual presence of an equimolar concentration of ATP that could be competing with ADP at these sites. Beyond this, it is necessary to realize that the site to which ADP binds in the exchange reaction undoubtedly differs from the site to which ATP binds to effect phosphorylation: ATP binds to an unphosphorylated enzyme, whereas ADP binds to the phosphorylated enzyme further along the reaction sequence. Recently, Fukushima and Tonomura [26] found that 0.2 mM ADP was at least as effective as 2 mM ADP in dephosphorylating an enzyme preparation (phosphorylated with 40 μM ATP).

CONCLUSIONS

These observations deny a unique role to Mg^{2+} in inhibiting the ADP/ATP exchange reaction. Moreover, recognition that Mg · ATP and Mg · ADP may inhibit exchange at concentrations corresponding to their affinities at substrate sites supports earlier formulations of an enzyme with two classes of nucleotide sites: high affinity sites at which Mg · ATP serves as substrate and exchange is catalyzed, and low affinity sites through which inhibition is produced by Mg · ATP, Mg · ADP, or Mg^{2+}. The two classes of sites could be located on the same polypeptide, although evidence that the catalytic units of the enzyme are present as dimers [25] supports the possibility that the two classes of sites exist on separate interacting units.

Such considerations may be accommodated in the following scheme (Fig. 4). In Reaction 1, Mg · ATP binds to the high affinity sites, a process that is inhibited competitively by ATP, ADP, and Mg · ADP. In Reaction 2, enzyme phosphorylation occurs. In Reaction 3, ADP (or Mg · ADP) dissociates from the enzyme. The ADP/ATP exchange reaction represents reversability over these three steps. It seems probable, although not demonstrated here, that ATP and/or Mg · ATP inhibit competitively in the reversal of Reaction 3. The studies of Fukushima and Tonomura [26], showing a slightly greater potency of free ADP compared to Mg · ADP in causing enzyme dephosphorylation, suggest that it is free ADP that dissociates and binds in the exchange reaction. Inhibition of the exchange reaction associated with a decrease in the ratio of ADP to Mg · ADP is also consistent with free ADP being the substrate in the reverse direction.

Release of inorganic phosphate, over a series of steps condensed into Reaction 4, represents the Na^+-dependent ATPase activity, a reaction catalyzed at the high affinity substrate sites [8].

Beyond competitions between ADP and ATP in Reactions 1 and 3, the exchange reaction also appears to be inhibited by concentrations of Mg · ATP, Mg · ADP, and free Mg^{2+} corresponding to their effective concentrations at the low

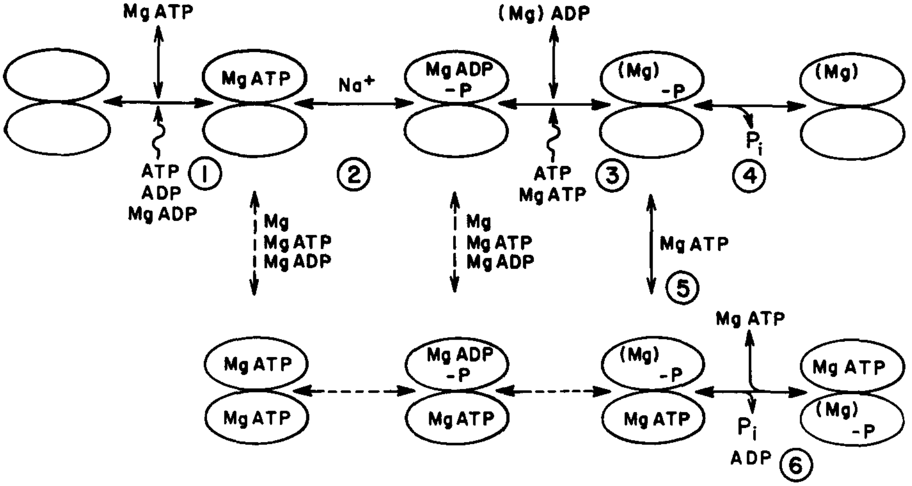

Fig. 4. Proposed reaction scheme. Addition of nucleotides to the high affinity site of the dimeric enzyme is shown in the upper reaction pathway, which participates in Na^+-dependent ADP/ATP exchange, Na^+-dependent ATPase activity, and the $(Na^+ + K^+)$-dependent ATPase activity at low substrate concentrations. Addition of Mg^{2+}, Mg · ADP, or Mg · ATP to the low affinity sites (vertical arrows) inhibits exchange by pulling the reaction process away from the steps involved in exchange. $(Na^+ + K^+)$-dependent ATPase activity at high ATP concentrations involves steps 1–6. Parentheses around Mg indicate uncertainty about its presence at that point.

affinity substrate sites of the enzyme. If the functioning of the $(Na^+ + K^+)$-dependent ATPase, at the concentrations of Mg · ATP found in vivo or usually added in vitro, involves participation of both the high and low affinity substrate sites [8], then the reaction course of the enzyme would include the addition of Mg · ATP, Reaction 5, perhaps following Reaction 3. Inhibition of the ADP/ATP exchange reaction would thus reflect the addition of Mg · ATP to the low affinity sites (Reaction 5), pulling the reaction away from steps 1 to 3 that constitute the exchange process. Moreover, inhibition would also occur with Mg · ADP or free Mg^{2+} binding to these same low affinity sites (where their affinity is comparable to that of Mg · ATP), similarly pulling the reaction away from the exchange steps.

For the overall ATPase reaction, step 6 depicts release of phosphate from the phosphorylated subunit, with subsequent phosphorylation of the alternate subunit by the second Mg · ATP bound. Successive steps would involve addition of a third Mg · ATP to the substrate site on the subunit first phosphorylated (now a low affinity site) and so forth. Nevertheless, data are not yet available that can distinguish between such a scheme for the ATPase, with catalysis alternating between sites, and formulations in which hydrolysis occurs solely at one class of sites, with the other class of sites, located either on the same or separate polypeptide, serving a regulatory function (e.g. stimulating the ATPase but inhibiting the exchange reaction).

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