Interactions of $Co(NH_3)_4ATP$ and $Cr(H_2O)_4ATP$ with Ca^{2+}-ATPase from Sarcoplasmic Reticulum and Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase from Kidney Medulla†

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ABSTRACT: In a series of steady-state kinetic studies, β,γ-bidentate $Cr(H_2O)_4ATP$ and $Co(NH_3)_4ATP$ have been found to be competitive inhibitors with respect to MnATP for the $(Na^+ + K^+)$-ATPase, the Mg^{2+}-ATPase, and the Ca^{2+}-ATPase. The K_i values for CoATP at both the (apparent) high- and low-affinity substrate sites for the Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase are very similar to the K_m values for MnATP obtained under the same conditions. At both substrate sites on the Ca^{2+}-ATPase, the K_i for CoATP was much weaker than the K_m for MnATP. CrATP was found to bind much more weakly than MnATP at the high-affinity substrate sites of the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-ATPase, while an almost equal affinity for MnATP and CrATP was observed at the high-affinity substrate site on the Mg^{2+}-ATPase. No low-affinity substrate site could be detected for the Ca^{2+}-ATPase with CrATP. At the low-affinity substrate sites of the Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase, however, the CrATP and MnATP appeared to have almost equal affinities. The use of ^{31}P NMR as a method for determining whether or not CoATP was a substrate was explored for the three ATPases. Both Ca^{2+}-ATPase and the Mg^{2+}-ATPase were observed to use CoATP as a substrate, while no substrate activity could be detected for the $(Na^+ + K^+)$-ATPase. Consistent with these ^{31}P NMR results is the observation that CoATP causes no inactivation of the $(Na^+ + K^+)$-ATPase. On the other hand, as previously demonstrated by Pauls et al. [Pauls, H., Bredenbrocker, B., & Schoner, W. (1980) *Eur. J. Biochem. 109*, 523] with the enzyme from beef brain and pig kidney, CrATP causes a slow inactivation of $(Na^+ + K^+)$-ATPase from sheep kidney.

Recent reports suggest a substantial similarity of structure and function among the various membrane-bound ATPase enzymes. Thus identical phosphorylated tripeptides have been isolated from the phosphoenzymes of both the plasma membrane $(Na^+ + K^+)$-ATPase[1] and sarcoplasmic reticulum Ca^{2+}-ATPase (Bastide et al., 1973). Chemical modification studies have provided evidence for cysteine (Patzelt-Wenczler et al., 1975; Champeil et al., 1978) and arginine (Depont et al., 1977; Grisham, 1979a; Murphy, 1976) residues at the nucleotide sites of both enzymes. However, the most intriguing similarities between these and other ATPases concern the nucleotide sites themselves. Thus kinetic studies on both the $(Na^+ + K^+)$-ATPase (Robinson, 1976) and Ca^{2+}-ATPase (Taylor and Hattan, 1979) indicate the existence of both high- and low-affinity ATP sites[2] on these enzymes during the catalytic cycle. Our own recent kinetic studies (Gantzer & Grisham, 1979b) demonstrate that a kidney Mg^{2+}-ATPase also exhibits both high- and low-affinity requirements for ATP. In all three cases, the apparent K_m at the high-affinity site is approximately 1–6 μM, while the apparent K_m at the low-affinity site is in the range of 0.5–8 mM. The precise nature and location of these sites at the active sites of these enzymes are not at all clear. For example, it has not been determined whether in any of these cases the two kinetically observed "sites" are in fact spatially distinct or whether they arise from a single site whose affinity for ATP changes during the catalytic cycle. In addition, the particular interactions between ATP and the requisite activating metal ions at either the high- or low-affinity "sites" on these enzymes have yet to be elucidated. Resolution of questions such as these will be greatly facilitated by the development of specific kinetic and spectroscopic probes. The substitution-inert complexes of ATP with Co(III) and Cr (III) are particularly useful in this regard (Cleland & Mildvan, 1979), and we have initiated a series of NMR and EPR studies of the interactions of these analogues with all three of the ATPases described above (Stephens & Grisham, 1979; Grisham, 1979b,c). In this paper, we present the results of a series of kinetic and NMR studies of these ATPases and their interactions with the Co(III) and Cr(III) analogues of ATP.

Experimental Procedures

Purifications. The Mg^{2+}-ATPase used in these studies was purified from sheep kidney as previously described (Gantzer & Grisham, 1979a). For the ^{31}P NMR studies, a very concentrated solution of enzyme was required. This was achieved by combining five to seven preparations of the purified enzyme and centrifuging the solution for 12 h at 58 000 rpm ($246000g_{av}$) in a Beckman 70 Ti rotor. As with the last centrifugation in the enzyme purification procedure, at the end of this centrifugation there are two layers visible in the centrifuge tube—a very dense reddish gold enzyme layer, with a large clear buffer layer above it. The Ca^{2+}-ATPase was

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[1] Abbreviations: TMA, tetramethylammonium; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; $(Na^+ + K^+)$-ATPase, sodium and potassium ion activated adenosinetriphosphatase; Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; Mg^{2+}-ATPase, magnesium ion activated adenosinetriphosphatase; CrATP, β,γ-bidentate $Cr(H_2O)_4ATP$; CoATP, β,γ-bidentate $Co(NH_3)_4ATP$.

[2] At this writing, it is not clear whether there exist two different substrate sites on these ATPases or whether the biphasic kinetics that are normally observed for these and other, similar ATPases are merely the result of a single ATP site with two different affinities for ATP in the course of the catalytic cycle. With this in mind, and for the sake of brevity, we use the terms "high-affinity substrate site" and "low-affinity substrate site". This point is discussed in detail under Discussion.

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purified from the white dorsal and leg muscles of freshly killed rabbits according to the procedure of MacLennan (1970). $(Na^+ + K^+)$-ATPase was purified from sheep kidney outer medulla membrane fragments (Schwartz et al., 1962) essentially according to the procedure of Jørgensen (1974).

Spectroscopic Methods. ^{31}P NMR experiments using the CoATP analogue were performed on a JEOL PS-100/EC 100 Fourier transform spectrometer at 40.48 MHz. Spectra were obtained by using broad-band proton decoupling on samples 1 mL in total volume. D_2O (20%) was added to the sample for field locking; samples were contained in 10-mm tubes. Spectra were accumulated by using a 45° pulse width, with a 1-s repetition rate.

For the substrate studies, the enzymes were incubated with 10 mM CoATP for 1 h at room temperature, and then EDTA was added to give a final EDTA concentration of 10 mM. (The actual incubation conditions are given in the figure legends.)

Preparation of Cr(III) and Co(III) Analogues. β,γ-Bidentate $Cr(H_2O)_4ATP$ was prepared according to Cleland & Mildvan (1979). The starting material for the synthesis of CoATP, $[Co(NH_3)_4CO_3]NO_3$, was prepared as described by Schlessinger (1960). The preparation of $Co(NH_3)_4ATP$ from $[Co(NH_3)_4CO_3]NO_3$ has been described by Cornelius et al. (1977).

Kinetic Studies. The ability of the two substrate analogues, $Co(NH_3)_4ATP$ and $Cr(H_2O)_4ATP$, to compete with Mn^{2+}-ATP at the ATP sites of the $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase was examined by using a ^{32}P assay procedure described previously (Gantzer & Grisham, 1979b). In all of these studies, Mn^{2+} was used as the divalent cation at a concentration equal to that of ATP plus 0.5 mM to assure that all of the ATP present was in the form of Mn^{2+}-ATP. The analogues were added to the assay mixture immediately prior to the addition of enzyme. The CoATP studies were conducted in 20 mM Tes–TMA, pH 7.5, and 25 °C, with the addition of 100 mM NaCl and 10 mM KCl for the $(Na^+ + K^+)$-ATPase studies and 0.1 mM $CaCl_2$ and 10 mM KCl for the Ca^{2+}-ATPase studies. The studies involving CrATP were carried out under the same conditions, with the exception that the buffer used was 20 mM Pipes, pH 6.0, since this analogue is not stable at higher pH values. In both cases, a racemic mixture of bidentate isomers was used.

In these kinetic studies, since the hydrolysis of MnATP is monitored by measuring the liberation of $^{32}P_i$ from Mn[γ-^{32}P]ATP, CoATP and CrATP will be observed to function as inhibitors. These analogues do, however, show substrate activity with these ATPases, as described below.

Results

Steady-State Kinetics of the Competition between Cr(III)- and Co(III)ATP and MnATP. The kinetic studies characterizing the effects of $Cr(H_2O)_4ATP$ and $Co(NH_3)_4ATP$ on the hydrolysis of MnATP[3] by the $(Na^+ + K^+)$-ATPase, the Mg^{2+}-ATPase and the Ca^{2+}-ATPase were plotted in the form of Dixon plots. This type of plot is useful for identifying the type of inhibition and for determining appropriate K_i values. In the case of competitive inhibition, the family of lines (where each line, in this case, is at a fixed MnATP concentration) in the Dixon plot intersects above the [I] axis in the second quadrant at $[I] = -K_i$. Replotting the y intercepts for each line in the form of a Lineweaver–Burk plot then allows determination of the apparent K_m for MnATP under the same experimental conditions, where the inhibitor concentration is extrapolated to zero.

Table I: Summary of Kinetically Determined Binding Constants[a]

	high affinity (μM)			low affinity (mM)		
	K_m	K_i	K_i/K_m	K_m	K_i	K_i/K_m
$Co(NH_3)_4ATP$ Kinetics						
$(Na^+ + K^+)$-ATPase	2.88	10	3.5	0.902	1.6	1.8
Mg^{2+}-ATPase	2.28	24	10.5	0.902	3.5	3.9
Ca^{2+}-ATPase	7.76	350	45.1	0.424	8.7	20.5
$Cr(H_2O)_4ATP$ Kinetics						
$(Na^+ + K^+)$-ATPase	10.7	256	23.9	0.368	0.46	1.3
Mg^{2+}-ATPase	7.4	16	2.2	0.19	0.17	0.89
Ca^{2+}-ATPase	10.4	545	52.4			

[a] The experimental data from which this table was derived (in the form of plots similar to Figure 1) are available as supplementary material (see paragraph at end of paper regarding supplementary material).

Both CoATP and CrATP proved to be competitive inhibitors with respect to MnATP in the presence of high or low levels of MnATP for all three enzymes. With the CoATP analogue and the $(Na^+ + K^+)$-ATPase, a K_i for CoATP at the high-affinity substrate site of 10 μM was obtained (Figure 1A). From the secondary plot of the y intercepts (Figure 1B), an apparent K_m for MnATP of 2.88 μM was obtained under the same experimental conditions. At the low-affinity substrate site for this enzyme, the K_i for CoATP was found to be 1.6 mM (Figure 1C), while the apparent K_m for MnATP was 0.902 mM (Figure 1D). Thus, at both substrate sites, the binding of the analogue appears to be slightly weaker than the binding of MnATP.

Similar results were obtained with the Mg^{2+}-ATPase, as shown in Table I. The Ca^{2+}-ATPase, on the other hand, appears to bind CoATP much more weakly than MnATP. In all cases, the binding of CoATP appeared to be weaker than the binding of MnATP. It is interesting to note that for all three ATPases, the ratio of K_i/K_m at the low-affinity site is smaller than the K_i/K_m ratio at the high-affinity site (Table I).

Similar results were obtained with the CrATP analogue in studies with the $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase, as shown in Table I. The binding of CrATP to the high-affinity site of the Ca^{2+}-ATPase was rather weak (K_i = 545 μM compared to a K_m for MnATP of 10.4 μM). Furthermore this K_i for CrATP was constant over a wide range of CrATP and MnATP concentrations, suggesting (1) that the interaction of CrATP with the Ca^{2+}-ATPase is different from that of CoATP and (2) that in this respect at least, metal–ATP complexes may interact with Ca^{2+}-ATPase in a manner different from that of the two other ATPases.

As with the CoATP analogue, the ratio of K_i/K_m for the CrATP at the low-affinity site for the $(Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase was lower than the same ratio at the high-affinity site. While the agreement between the K_m values for MnATP in the CrATP and CoATP experiments is generally good, it may be the case that some of the differences between these two sets of data may be due in part to the difference in pH in these studies. A pH of 7.5 was used for the CoATP studies, while a pH of 6.0 was used for the CrATP experiments. The lower pH is required in the latter case due

[3] Although Mg^{2+} is the predominant activator of these ATPases in vivo, Mn^{2+} was used in the present studies, since (1) Mn^{2+} and Mg^{2+} are very similar in their activation of these enzymes, (2) Mn^{2+} is a useful paramagnetic probe of these enzymes (Gantzer & Grisham, 1979b; Grisham & Mildvan, 1974; O'Connor & Grisham, 1979, 1980a,b), and (3) we have already accumulated a large amount of data on the activation of these enzymes by Mn^{2+} (Gantzer & Grisham, 1979b; Grisham & Mildvan, 1974).

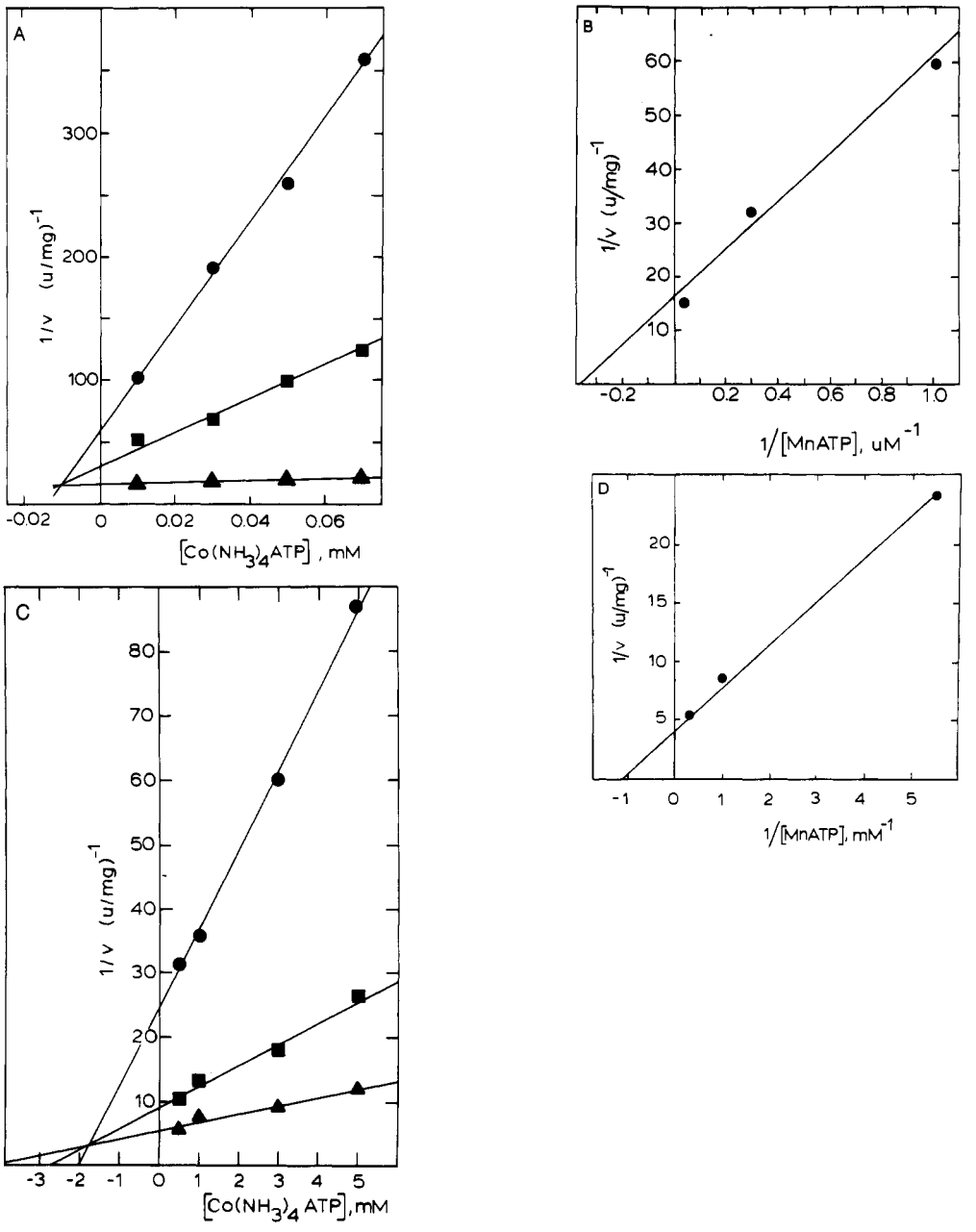


FIGURE 1: (A) Dixon plot of CoATP inhibition of MnATP hydrolysis at the high-affinity substrate site of the $(Na^+ + K^+)$-ATPase. Conditions and methods are as described in the text. The ATP concentrations were as follows: (●) 1 μM; (■) 3 μM; (▲) 30 μM. The measured K_i was 10 μM. (B) Lineweaver–Burk plot of the y intercepts of (A). The extrapolated K_m for MnATP was determined to be 2.88 μM. (C) Dixon plot of CoATP inhibition of MnATP hydrolysis at the low-affinity substrate site of the $(Na^+ + K^+)$-ATPase. Conditions and methods are as described in the text. The ATP concentrations were as follows: (●) 0.2 mM; (■) 1.0 mM; (▲) 3.0 mM. The K_i was determined to be 1.6 mM. (D) Lineweaver–Burk plot of the y intercepts of (C). The extrapolated K_m for MnATP is 0.902 mM.

to the instability of CrATP at pH values higher than 6 (Dunaway-Mariano & Cleland, 1980a).

Slow Inactivation of $(Na^+ + K^+)$-ATPase by CrATP. Recently, Pauls et al. (1980) demonstrated that CrATP causes a slow inactivation of the $(Na^+ + K^+)$-ATPase from beef brain and pig kidney. These studies are complementary to the steady-state kinetic studies described in this paper. Thus the K_D for the enzyme–CrATP complex measured in the presence of 3 mM KCl by Pauls et al. (0.33 mM) agrees well with the K_i values reported here for the $(Na^+ + K^+)$-ATPase. It should be noted that at the temperature employed here for the steady-state kinetics, and over the time required for the enzyme assays (0.5–10 min), no significant inactivation of the $(Na^+ + K^+)$-ATPase or the other ATPases was observed. On the other hand, as shown in Figure 2, sheep kidney $(Na^+ + K^+)$-ATPase is inactivated by extended exposure to CrATP at 37 °C, in a manner similar to that described by Pauls et al. for the beef brain and pig kidney enzymes. For reasons that will become obvious later in this paper, it was of interest to us to see if $Co(NH_3)_4ATP$ could likewise inactivate the $(Na^+ + K^+)$-ATPase. As shown in Figure 2, the Co(III) analogue causes no detectable inactivation after a 40-min incubation. Beyond this point, spontaneous breakdown of $Co(NH_3)_4ATP$ releases significant amounts of Co(III), which causes some inactivation of the ATPase. This latter process has previously been characterized in our laboratory (McClaugherty & Grisham, 1982).

^{31}P NMR Studies of CoATP Hydrolysis by ATPases. In order to examine further the interaction of $Co(NH_3)_4ATP$ with the three ATPases, a series of ^{31}P NMR studies were carried out. The 40.5-MHz ^{31}P NMR spectrum of $Co(NH_3)_4ATP$ at pH 7.5 is shown in Figure 3A. The peak assignments

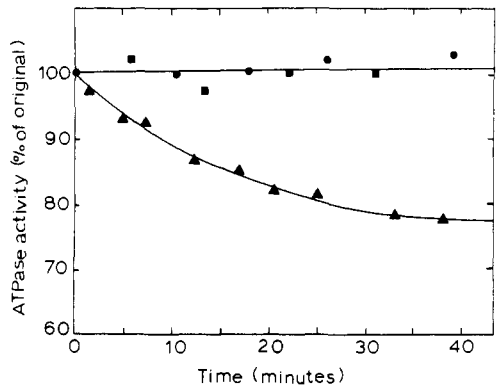


FIGURE 2: Effects of incubation with β,γ-bidentate $Cr(H_2O)_4ATP$ and β,γ-bidentate $Co(NH_3)_4ATP$ on sheep kidney $(Na^+ + K^+)$-ATPase. Incubation solutions contained 83 mM imidazole hydrochloride pH 7.25, 18% (w/v) sucrose, and (▲) 0.19 mM CrATP, (■) 0.18 mM CoATP, or (●) no other additions. Enzyme concentration was 0.4 mg/mL in all incubations. At the times shown, aliquots were withdrawn and assayed as previously described (Grisham & Mildvan, 1974). Temperature = 37 °C in both incubation and assay.

shown are those originally made by Cornelius et al. (1977). Compared to free ATP, the β-P and α-P resonances are shifted 10.4 and 9.9 ppm downfield, respectively, due to coordination with the Co(III), while the uncoordinated α-P is shifted 0.4 ppm upfield.

In the hope of using ^{31}P NMR to examine the substrate activity of $Co(NH_3)_4ATP$ with the ATPases, we first considered hexokinase, an enzyme that is known to use $Co(NH_3)_4ATP$ as a substrate. Cornelius & Cleland (1978) showed that, in the presence of hexokinase and glucose, $Co(NH_3)_4ATP$ is slowly converted to $Co(NH_3)_4ADP$-glucose 6-phosphate. A 1-h incubation of $Co(NH_3)_4ATP$ with hexokinase and glucose causes a slow broadening of the ^{31}P resonances of $Co(NH_3)_4ATP$ and also results in the appearance of doublets at 1.2 and 12.2 ppm. These resonances correspond to the α-P of Co(III)-coordinated ADP (Cornelius et al., 1977) and Co(III)-coordinated glucose 6-phosphate, respectively. The doublet arising from the β-P of the ADP product is buried at 4.3 ppm with the γ-P of $Co(NH_3)_4ATP$.

A similar incubation of the Ca^{2+}-ATPase with $Co(NH_3)_4ATP$ (Figure 3B) resulted in a dramatic, time-dependent broadening of the ^{31}P resonances of $Co(NH_3)_4ATP$. The broadening was reversed by EDTA and is due to the release of Co(III), with subsequent reduction to paramagnetic Co(II).[4] Concomitant with this broadening was the appearance of a signal at −0.4 ppm, corresponding to inorganic phosphate, the expected product of ATP hydrolysis by Ca^{2+}-ATPase. The absence of peaks corresponding to either $Co(NH_3)_4ADP$ or free ADP is consistent with the fact that many ATPases use ADP (slowly) as a substrate, producing AMP and inorganic phosphate as products (Laliberté et al., 1982; Cori et al., 1965; Gantzer, 1980; Grisham, 1979c).[5] The

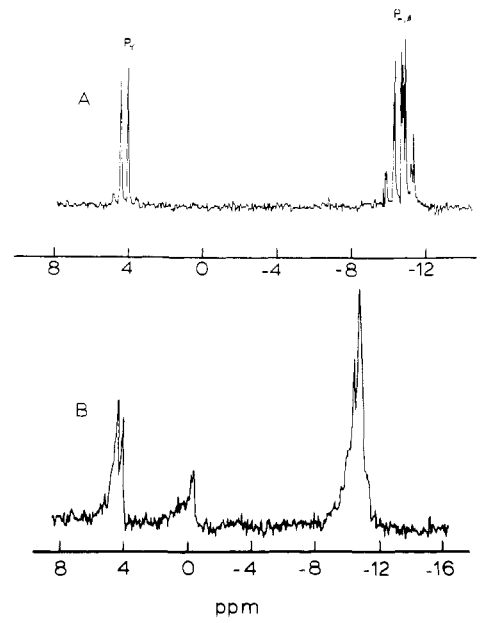


FIGURE 3: ^{31}P NMR spectrum at 40.48 MHz of β,γ-bidentate $Co(NH_3)_4ATP$. 1000 scans were collected on a 25 mM CoATP solution, pH 7.5, in a 10-mm sample tube, as described under Experimental Procedures. (B) ^{31}P NMR spectrum obtained after incubation of $Co(NH_3)_4ATP$ with Ca^{2+}-ATPase. Incubation solution contained 10 mM CoATP, 20 mM Tes–TMA, pH 7.5, 10 mM KCl, 0.1 mM $CaCl_2$, and 50 μM Ca^{2+}-ATPase. 4000 scans were collected following a 1-h incubation at 23 °C as described under Experimental Procedures.

^{31}P resonance of AMP produced in this way would be at 0.3 ppm, and indeed a broad resonance is observed at this position in Figure 3B. The incubation of $Co(NH_3)_4ATP$ with Mg^{2+}-ATPase yields a ^{31}P spectrum that is similar to that obtained with the Ca^{2+}-ATPase. However, similar incubations of $Co(NH_3)_4ATP$ with $(Na^+ + K^+)$-ATPase produced no changes in the ^{31}P spectrum, indicating that $Co(NH_3)_4ATP$ is not a substrate for $(Na^+ + K^+)$-ATPase.

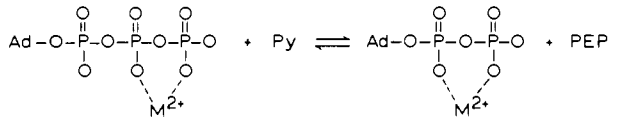
Discussion

The steady-state kinetic studies of Figure 1 establish that the Co(III) and Cr(III) analogues of ATP compete effectively with MnATP with all three of the ATPases examined. While the inhibition data were presented in the form of Dixon plots in Figure 1, it should be pointed out that double-reciprocal plots ($1/v$ vs. $1/[MnATP]$) of these same data are also consistent with competitive inhibition of all three ATPases by $Co(NH_3)_4ATP$ and $Cr(H_2O)_4ATP$. The observation of biphasic kinetics in these inhibition studies is entirely consistent with previous work in numerous laboratories with these and other ATPases. Thus, manifestations of both high- and low-affinity requirements for ATP and/or metal–ATP have been described for $(Na^+ + K^+)$-ATPase (Kanazawa et al., 1970; Robinson, 1976), Ca^{2+}-ATPase (Taylor & Hattan, 1979), Mg^{2+}-ATPase (Gantzer & Grisham, 1979b), mitochondrial (F_1) ATPase (Takeshige et al., 1976), and myosin ATPase (Yee et al., 1980). There are several possible causes for these kinetic patterns, including (1) heterogeneity of ATPase reaction sites (for example, a mixture of native and altered active sites), each class of sites characterized by its own V_{max} and K_m, (2) allosteric coupling between active sites, so that saturation of one site has a negative effect on ATP binding and a positive effect on ATP hydrolysis, and (3) allosteric coupling between an active site and an effector site that binds ATP. In previous studies of biphasic ATPase reaction kinetics, numerous workers have presented evidence ruling out heterogeneity of reaction sites with the ATPases described here

[4] J. M. Van Divender and C. M. Grisham, unpublished observations.

[5] The production of AMP could of course also be due to contamination with adenylate kinase (myokinase) that, together with an ATPase, would convert a pool of ATP [or $Co(NH_3)_4ATP$] to AMP and phosphate. We have ruled out the possibility of adenylate kinase contamination using diadenosine pentaphosphate (Ap_5A), a potent inhibitor of adenylate kinase. Solutions of the ATPase (10 μM) were incubated for 1 h at 23 °C in 20 mM Pipes, pH 6.0, 10 mM KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, and either 10 mM $Co(NH_3)_4ATP$ or 10 mM ATP. Identical NMR spectra are obtained in the presence and absence of 10 μM Ap_5A. Adenylate kinase assays under these conditions demonstrate that (a) adenylate kinase is 92–95% inhibited under these conditions and that (b) no adenylate kinase is detectable in the Ca^{2+}-ATPase preparations used for the NMR studies.

Scheme I

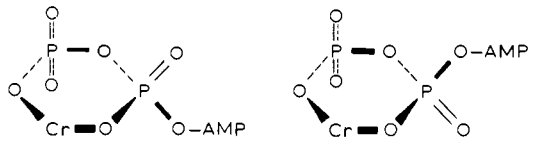


(Cantley et al., 1978; Gantzer & Grisham, 1979b; Taylor & Hattan, 1979; Neet & Green, 1977). Perhaps the most compelling arguments have been raised by Taylor & Hattan (1979) in studies of the SR Ca^{2+}-ATPase. In this latter work, the effects of the ATP analogue AMP-PCP clearly rule out active site heterogeneity as a cause of the observed biphasic response to ATP. Moreover, they demonstrate that both phases of the ATP rate curve respond similarly to variations in pH, temperature, nucleotide substrate, metal ion transported, and product inhibition by ADP. Also, Neet & Green (1977), who concluded that chemical heterogeneity of active sites was unlikely, found that the biphasic behavior of the Ca^{2+}-ATPase was unaffected by a variety of Ca^{2+} levels and by detergent extraction of the ATPase. Entirely analogous studies have been carried out with the $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase with the same results (Grisham, 1979a–c; Gantzer & Grisham, 1979b). Remaining from the mechanisms suggested above are the allosteric mechanisms and a number of these have been proposed, including allosteric substrate activation (Taylor & Hattan, 1979; Yee et al., 1980) and alternating site cooperativity (Hackney & Boyer, 1978). A central question for all of these mechanisms concerns whether these apparent high- and low-affinity requirements for ATP arise from two distinct sites or whether they result from a single site, whose affinity for ATP changes during the catalytic cycle. Among the many groups that have addressed this question, Cantley et al. (1978), for example, suggested that two sites exist simultaneously on the $(Na^+ + K^+)$-ATPase. However, more recent studies by the same group (Smith et al., 1980) are interpreted in terms of a single-site model. It would in fact appear that the evidence for neither model is compelling as it presently stands. Furthermore, kinetic methods alone may never be able to resolve this question. A judicious combination of structural studies and kinetics may eventually be more useful.

On the other hand, the role and eventual fate of divalent cations with these ATPases are better understood at this writing. For example, the bulk of the evidence suggests that ATP acts as a substrate for these enzymes as a metal–ATP complex. Recent evidence, both kinetic and spectroscopic, suggests that in fact two divalent metals are required at the active site of the $(Na^+ + K^+)$-ATPase, one bound to ATP and one bound directly to the enzyme (Grisham, 1981; O'Connor & Grisham, 1980b). The present work demonstrates that the complexes of trivalent Cr(III) and Co(III) with ATP are good analogues for the metal–ATP substrate complex for all three enzymes examined. The coordination of metal and the ATP in the analogues used here is β,γ-bidentate, and the reasonable agreement between K_i and K_m values in Table I could be interpreted as an indication that the β,γ-bidentate metal–ATP complex is the preferred substrate for these enzymes. Of the numerous enzymes examined to date, none use tridentate CrATP or CoATP as substrates (Dunaway-Mariano & Cleland, 1980b; Cleland & Mildvan, 1979).

Compared to the attention devoted to the nature of the substrate for these enzymes, the nature of the products has received little attention. If metal–ATP is in fact the substrate for these enzymes, the ADP product could be released from the enzyme either as the free nucleotide or as the metal–ADP complex. Precedent for this latter case has been established

Chart I



Δ-$Cr(H_2O)_4$ATP Λ-$Cr(H_2O)_4$ATP

with pyruvate kinase (Mildvan, 1979), which uses β,γ-bidentate metal–ATP as a substrate for the phosphorylation of pyruvate and releases α,β-bidentate metal–ADP as a product as shown in Scheme I. An analogous path apparently obtains for hexokinase, as evidenced by the appearance of peaks for glucose 6-phosphate and α,β-bidentate CoADP in the NMR studies described above.

If the same mechanism applied to the Ca^{2+}-ATPase or Mg^{2+}-ATPase, one should observe peaks for CoADP in the ^{31}P NMR spectra of Figure 3 above. However, peaks for CoADP are noticeably absent from the spectra. In the case of the Ca^{2+}-ATPase, the NMR data may be compared with the recent kinetic studies of Yamada & Ikemoto (1980), who suggest that the product of the Ca^{2+}-ATPase reaction is free ADP, not metal–ADP. Similar data are not available for the Mg^{2+}-ATPase.

Although no hydrolysis of CoATP could be detected with the $(Na^+ + K^+)$-ATPase, the β,γ-bidentate CrATP is in fact a substrate for the $(Na^+ + K^+)$-ATPase. Circular dichroism experiments indicate that this enzyme utilizes the Δ isomer of CrATP, shown in Chart I (Gantzer, 1980). In a similar manner, it has been shown that the Ca^{2+}-ATPase utilizes the Δ isomer of CrATP, while the Mg^{2+}-ATPase utilizes the Λ isomer. These results will be described in detail in a forthcoming paper.

Evidence for the hydrolysis of CrATP by the $(Na^+ + K^+)$-ATPase has also been obtained by Pauls et al. (1980). In these studies, the slow inactivation of the $(Na^+ + K^+)$-ATPase is shown to occur concomitantly with ATP hydrolysis. The inactivation of the enzyme is shown to result from the formation of a stable complex of phosphorylated enzyme and Cr(III). Whereas Pauls et al. made no effort to determine the partitioning between the hydrolysis and inactivation pathways, a comparison of the previously described circular dichroism studies and the inactivation data of Figure 2 indicates that the $(Na^+ + K^+)$-ATPase, on average, hydrolyzes 200 mol of CrATP/mol of enzyme before being inactivated.

The present work supports the conclusion of Pauls et al. that inactivation is preceded by hydrolysis of CrATP. Thus, from the failure to observe hydrolysis of CoATP with the $(Na^+ + K^+)$-ATPase, one would anticipate that little if any inactivation of this enzyme would occur upon prolonged incubation with CoATP. This is what is observed in Figure 2. The ability of the $(Na^+ + K^+)$-ATPase to use CrATP as a substrate, but not CoATP, is not without precedent in the literature. Thus it has been shown (Gupta et al., 1976) that the enolization of pyruvate by pyruvate kinase is promoted by CrATP but not by CoATP. For both of these enzymes, this difference may be due simply to the added bulk of the ammine ligands of $Co(NH_3)_4$ATP relative to the water ligands of $Cr(H_2O)_4$ATP.

Supplementary Material Available

Plots of the experimental data (21 pages). Ordering information is given on any current masthead page.

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