Binding to the High-Affinity Substrate Site of the (Na$^+$ + K$^+$)-Dependent ATPase

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Abstract

The (Na$^+$ + K$^+$)-dependent ATPase exhibits substrate sites with both high affinity (K_m near 1 μM) and low affinity (K_m near 0.1 mM) for ATP. To permit the study of nucleotide binding to the high-affinity substrate sites of a canine kidney enzyme preparation in the presence as well as absence of MgCl$_2$, the nonhydrolyzable β-γ imido analog of ATP, AMP-PNP, was used in experiments performed at 0–4°C by a centrifugation technique. By this method the K_D for AMP-PNP was 4.2 μM in the absence of MgCl$_2$. Adding 50 μM MgCl$_2$, however, decreased the K_D to 2.2 μM; by contrast, higher concentrations of MgCl$_2$ increased the K_D until, with 2 mM MgCl$_2$, the K_D was 6 μM. The half-maximal effect of MgCl$_2$ on increasing the K_D occurred at approximately 1 mM. This biphasic effect of MgCl$_2$ is interpreted as Mg^{2+} in low concentrations favoring AMP-PNP binding through formation at the high-affinity substrate sites of a ternary enzyme–AMP-PNP–Mg complex; inhibition of nucleotide binding at higher MgCl$_2$ concentrations would represent Mg^{2+} acting through the low-affinity substrate sites. NaCl in the absence of MgCl$_2$ increased AMP-PNP binding, with a half-maximal effect near 0.3 mM; in the presence of MgCl$_2$, however, NaCl increased the K_D for AMP-PNP. KCl decreased AMP-PNP binding in the presence or absence of MgCl$_2$, but the simultaneous presence of a molar excess of NaCl abolished (or masked) the effect of KCl. ADP and ATP acted as competitors to the binding of AMP-PNP, although a substrate for the K$^+$-dependent phosphatase reaction also catalyzed by this enzyme, p-nitrophenyl phosphate, did not. This lack of competition is consistent with formulations in which the phosphatase reaction is catalyzed at the low-affinity substrate sites.

Introduction

The (Na$^+$ + K$^+$)-dependent ATPase, the enzymatic basis of the plasma membrane sodium pump [1, 2, 3], has two classes of sites for ATP apparent in kinetic studies: high-affinity substrate sites with a K_m near 1 μM, and low-affinity substrate sites with a K_m near 0.1 mM [4–7]. In such studies

165

$MgCl_2$ is present as a requirement for enzymatic activity. Although it is not technically feasible to measure binding to the low-affinity substrate sites, experiments measuring directly the binding of ATP to the high-affinity substrate sites revealed a K_D of 0.1–0.2 μM [8, 9]. In these binding studies, however, it was necessary to exclude Mg^{2+} to prevent hydrolysis of ATP. Since Mg^{2+} may well interact with ATP at the substrate sites, and since *in vivo* and under usual assay conditions *in vitro* essentially all the ATP is present as the Mg–ATP complex, this limitation to the binding studies is troubling. Thus it seemed of interest to reexamine binding to the high-affinity substrate sites using a nonhydrolyzable analog of ATP, β-γ imido ATP (AMP-PNP), which permits experiments in the presence of $MgCl_2$ as well as its absence. This report describes such studies, showing that $MgCl_2$ can either enhance or diminish nucleotide binding to the high-affinity substrate sites, depending on its concentration. Moreover, the presence of $MgCl_2$ also modifies the response of nucleotide binding to another mechanistically and physiologically important ligand, Na^+.

Methods

The enzyme preparation was obtained from the medullae of frozen canine kidneys by a modification [10] of the procedure of Jorgensen [11]; ATP present in the preparation was removed by incubation at 37°C and subsequent washing by centrifugation, as described by Schoot et al. [12]. Purity, estimated by sodium dodecylsulfate gel electrophoresis, was approximately 85%.

AMP-PNP binding was measured by adding at 0°C the enzyme preparation (0.1–0.5 mg protein), 30 mM histidine · HCl-Tris (pH 7.8), and various concentrations of ^{3}H-AMP-PNP, together with other ligands as indicated. EDTA, 0.1 mM, was present when $MgCl_2$ was omitted. The suspension was mixed twice with a vortex agitator for 15 sec each with a 1-min cooling interval between, and then centrifuged at 0–4°C for 30 min at 110,000 g. The supernatant material was decanted, and the interior of the tube was wiped carefully, twice with damp and twice with dry cotton swabs, up to the margins of the pellet. The pellet was then dissolved in 0.5 ml 2 N NaOH by heating overnight at 65°C, and this solution next neutralized with HCl. Radioactivity was measured using a Beckman liquid scintillation counter equipped with automatic quench control. "Nonspecific binding" was estimated, for each experimental condition, by parallel incubations containing in addition 0.5 mM unlabeled AMP-PNP; this "nonspecific binding" was

subtracted from the total binding observed in the absence of the 0.5 mM
AMP-PNP to give the specific binding, which is reported under Results. The
"nonspecific binding," which also includes ^{3}H-AMP-PNP trapped in the
pellet, ranged from 10 to 30% of the total binding.

Protein was determined by the Lowry method [13].

^{3}H-AMP-PNP and unlabeled AMP-PNP were purchased from ICN
Chemical and Radioisotope Division; ATP, ADP, and p-nitrophenyl phos-
phate were purchased from Sigma Chemical Co.

Data presented are averages of four determinations, each representing
the average of triplicate incubations.

Results

The K_D for AMP-PNP binding, measured at 0–4°C in the absence of
MgCl$_2$ (presence of 0.1 mM EDTA), was 4.2 μM (Fig. 1A). The maximal
binding was 1 mole of AMP-PNP per 300–350,000 g of protein, consistent
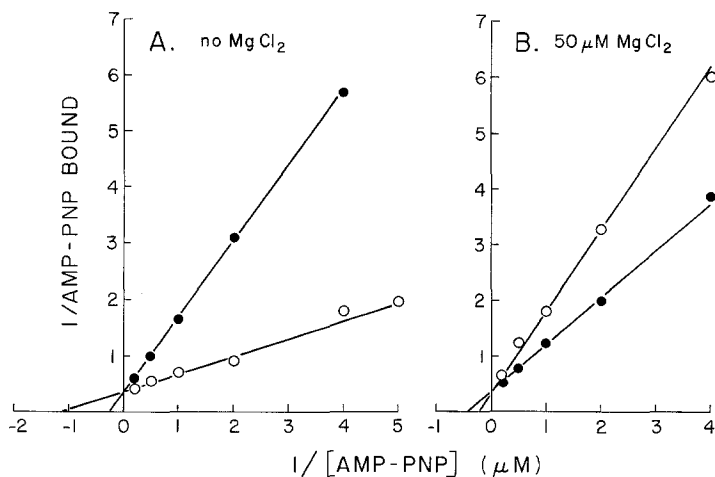with one high-affinity substrate binding site per functional enzyme complex,

Fig. 1. Effects of MgCl$_2$ and NaCl on AMP-PNP binding. The specific binding of AMP-PNP,
over the range of concentrations indicated, was measured as described under Methods. In panel
A, experiments in the absence of added MgCl$_2$ (and the presence of 0.1 mM EDTA) are plotted
in double-reciprocal form, for incubations in the absence of NaCl (●) or presence of 10 mM
NaCl (○). In panel B, experiments in the presence of 50 μM MgCl$_2$ are plotted similarly, for
incubations in the absence of NaCl (●) or presence of 10 mM NaCl (○). The amount of binding
is expressed relative to that with 2 μM AMP-PNP, and no NaCl or MgCl$_2$, defined as 1.0.

composed of two catalytic or alpha subunits and two (or four) glycoprotein or beta subunits [1–3]. Because of some variation in absolute binding capacity between different enzyme preparations, data are normalized to the binding in the presence of 2 μM AMP-PNP defined as 1.0; with this scale the maximal binding was 2.5 normalized units (Fig. 1A).

Addition of 50 μM MgCl$_2$ to the incubation medium decreased the K_D to 2.2 μM (Fig. 1B), without affecting the maximal binding capacity. Further additions of MgCl$_2$, however, progressively increased the K_D for AMP-PNP, until with 2 mM MgCl$_2$ the K_D was 6 μM (Fig. 2).

These effects of MgCl$_2$ on the K_D for AMP-PNP were modified by including in the incubation mixture low concentrations of NaCl. With 10 mM NaCl, the K_D measured in the absence of MgCl$_2$ was markedly decreased to 0.9 μM (Fig. 1A). On the other hand, in the presence of 50 μM MgCl$_2$, this concentration of NaCl increased the K_D for AMP-PNP from 2.2 μM to 4.2 μM (Fig. 1B). Further additions of MgCl$_2$ progressively increased the K_D so that with 2 mM MgCl$_2$ the K_D was 12 μM (Fig. 2). Thus, MgCl$_2$ decreased the K_D for AMP-PNP only in the absence of NaCl, and even in that case only at concentrations below 1 mM. Moreover, NaCl decreased the K_D only in the absence of MgCl$_2$.

The ability of NaCl to alter the binding of AMP-PNP varied with its concentration; with 1 μM AMP-PNP (and in the absence of MgCl$_2$) the half-maximal increment in binding occurred near 0.3 mM (Fig. 3).

Previous studies (performed in the absence of MgCl$_2$) indicated that KCl decreased the binding of ATP [8, 9], in accord with kinetic studies (performed in the presence of MgCl$_2$) which showed that KCl increased the apparent K_m for ATP [14, 15]. KCl also decreased the binding of AMP-PNP, acting as a competitive inhibitor. In the absence of MgCl$_2$ the K_i for
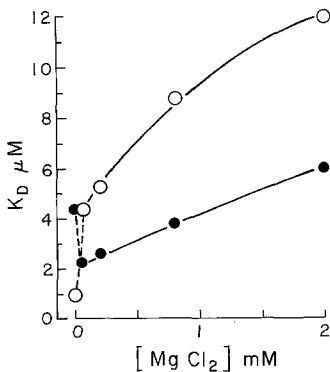

Fig. 2. Effect of MgCl$_2$ and NaCl on the K_D for AMP-PNP binding. Values of K_D for AMP-PNP binding, determined as in Fig. 1, are plotted against the concentrations of MgCl$_2$ added to the medium, for incubations in the absence of NaCl (●) or the presence of 10 mM NaCl (O).
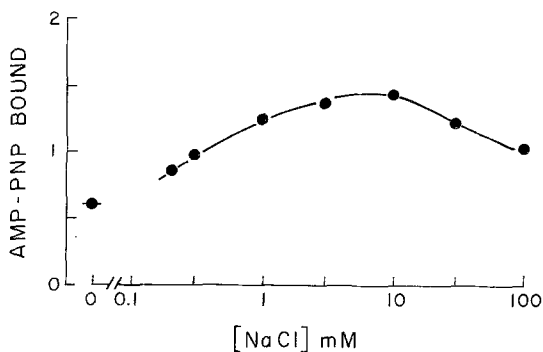
Fig. 3. Effect of NaCl concentration on AMP-PNP binding. The specific binding of AMP-PNP is plotted against the concentration of NaCl added to the medium, in experiments with 1 μM AMP-PNP and no added MgCl$_2$ (presence of 0.1 mM EDTA).

KCl, estimated from Dixon plots, was 0.5 mM (Fig. 4). A similar value was obtained in the presence of 1 mM MgCl$_2$ (data not shown).

When NaCl and KCl were added together, the effects of NaCl antagonized (or masked) any effects of KCl so long as NaCl was in substantial molar excess. Thus, in the absence of MgCl$_2$, 10 mM NaCl prevented the decrease in AMP-PNP binding due to 1 mM KCl, and led to a level of AMP-PNP binding expected with NaCl alone (Table I). In the presence of 1 mM MgCl$_2$, conditions under which NaCl and KCl each separately decreased binding of AMP-PNP, the simultaneous presence of 10 mM NaCl and 1 mM KCl again led to a level of AMP-PNP binding expected with NaCl
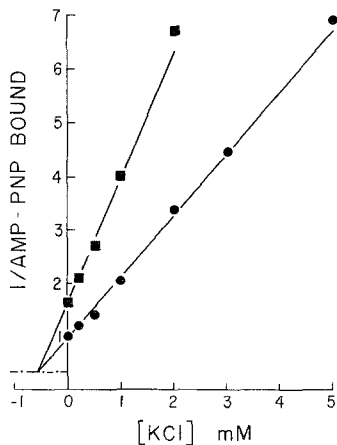

Fig. 4. Effect of KCl on AMP-PNP binding. The specific binding of AMP-PNP is plotted against the concentration of KCl added to the medium, from experiments with 2 μM (●) or 1 μM (■) AMP-PNP, all in the absence of added MgCl$_2$ (presence of 0.1 mM EDTA). Data are presented in the form of a Dixon plot; the interrupted horizontal line represents the reciprocal of the maximal binding.

Table I.   Effects of NaCl and KCl on AMP-PNP Binding[a]

	Relative binding of AMP-PNP	
Incubation conditions	Without MgCl$_2$	With 1 mM MgCl$_2$
No NaCl or KCl	1.0	0.96
NaCl, 10 mM	1.9	0.54
KCl, 1 mM	0.49	0.50
NaCl plus KCl	1.8	0.51

[a]Specific binding of AMP-PNP was measured, as described under Methods, in incubations with 2 μM AMP-PNP and the concentrations of NaCl and KCl indicated, in the absence of MgCl$_2$ (presence of 0.1 mM EDTA) or the presence of 1 mM MgCl$_2$. Binding is presented relative to that in the absence of MgCl$_2$, NaCl, and KCl, defined as 1.0.

alone (Table I). Similar results were obtained with 100 mM NaCl and 10 mM KCl (data not shown).

On this basis, the K_i for AMP-PNP as a competitor toward ATP, calculated previously as 3 μM from kinetic studies in the presence of 90 mM NaCl, 10 mM KCl, and 500 μM free Mg^{2+} with a different enzyme preparation [5], is in fair agreement with the K_D for AMP-PNP binding estimated from Fig. 2 for analogous conditions, 7 μM.

The effects of ATP on AMP-PNP binding were also measured for comparison with earlier studies on ATP binding (and, as in studies on ATP binding, only measurements in the absence of MgCl$_2$ are meaningful because of the small but finite hydrolysis of ATP that occurs, even at 0–4°C, during processing). ATP acted as a competitor toward AMP-PNP, with a K_i in the presence of 10 mM NaCl of 0.3 μM (Fig. 5B), and in absence of NaCl of 0.6 μM (Fig. 5A). This latter value is somewhat larger than, although of the same magnitude as, the K_D values previously reported for ATP binding, 0.1–0.2 μM [8, 9].

ADP also was a competitor toward AMP-PNP binding, with a K_i of 2.5 μM in the absence of MgCl$_2$; this K_i was increased to 8 μM in the presence of 200 mM MgCl$_2$ (data not presented). Earlier studies [9, 16] on ADP as a competitor to ATP binding (in the absence of MgCl$_2$) reported a K_i of 1–2 μM, while kinetic studies [5] of ATPase activity (in the presence of MgCl$_2$) revealed a K_i for ADP of 8 μM. Direct measurements of ^{14}C-ADP binding in the presence of 5 mM NaCl revealed a K_D of 0.3 μM, with MgCl$_2$ antagonizing this binding [17].

In contrast to these competitions for binding between AMP-PNP and either ATP or ADP, a substrate for the K$^+$-phosphatase reaction that is also catalyzed by this enzyme, p-nitrophenyl phosphate, was a poor inhibitor. Thus, with 0.6 μM AMP-PNP, in the absence of NaCl, KCl, and MgCl$_2$, the inclusion of 2 mM p-nitrophenyl phosphate decreased binding of AMP-PNP
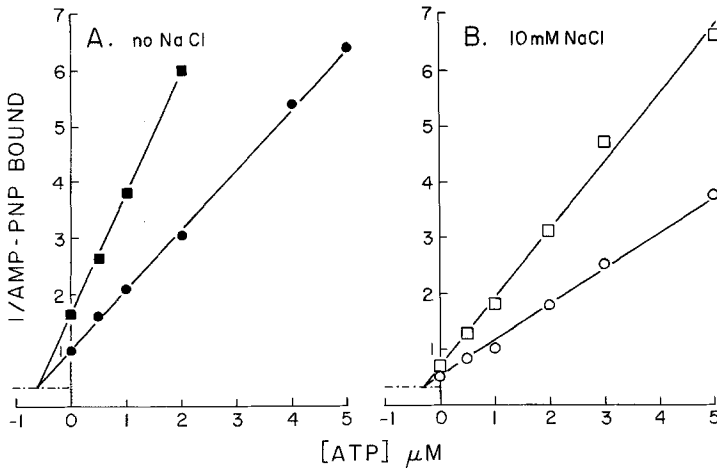
Fig. 5. Effect of ATP on AMP-PNP binding. In panel A experiments measuring specific binding of AMP-PNP are presented in the form of a Dixon plot as in Fig. 4, from incubations in the presence of the concentrations of ATP indicated and either 2 μM (●) or 1 μM (■) AMP-PNP. No NaCl or MgCl₂ was added. In panel B corresponding experiments in the presence of 10 mM NaCl and either 2 μM (○) or 1 μM (□) AMP-PNP are presented. Again, no MgCl₂ was added.

only 10 ± 4%; since the K_m for p-nitrophenyl phosphate in the K⁺-phosphatase reaction is 3 mM [5] and the K_D for AMP-PNP is 4.2 μM under these conditions, an inhibition of 37% would be expected with p-nitrophenyl phosphate if it were a competitor.

Discussion

Substitution of the nonhydrolyzable analog AMP-PNP for ATP in studies of binding to the high-affinity substrate sites of the (Na⁺ + K⁺)-ATPase allows measurements in the presence as well as absence of MgCl₂. Thus the dilemma resulting from kinetic studies of substrate binding requiring the presence of MgCl₂ (to permit enzymatic activity) and from binding studies requiring the absence of MgCl₂ (to avoid hydrolysis of ATP) may be circumvented. Previous kinetic studies [5] indicated that AMP-PNP behaves as a competitor toward ATP at the high-affinity substrate sites, with a K_i comparable to the K_D found here in the presence of the ligands necessary for ATPase activity (Na⁺, K⁺, and Mg²⁺). Conversely, the K_i for ATP as a competitor to AMP-PNP binding reported here (necessarily measured in the absence of MgCl₂) is comparable to previous reports [8, 9] of the K_D for ATP (also measured in the absence of MgCl₂). On these grounds, then, the use of

AMP-PNP may be justified as an analog at the high-affinity substrate sites. Moreover, AMP-PNP seems preferable to another possible analog, ADP, not only in its more complete structural resemblance to ATP but also in its closer resemblance in terms of Mg-binding [18].

By using AMP-PNP it was thus possible to demonstrate specific interrelationships between nucleotide binding and the activating cations. Most notably, $MgCl_2$, in the absence of NaCl, first decreased and then increased the K_D for AMP-PNP. The concentration dependence of this biphasic effect suggests that the decrease in K_D occurs when the level of $MgCl_2$ is comparable to the K_D for AMP-PNP filling the high-affinity sites. Thus, formation of the ternary complex enzyme–AMP-PNP–Mg results in a tighter binding of AMP-PNP than in the binary complex enzyme–AMP-PNP. Since studies of Mg binding to the enzyme do not reveal a K_D for free Mg^{2+} in the micromolar range [19], these data indicate that a major aspect of Mg binding to the enzyme is through the nucleotide, consistent with the high stability of the Mg–nucleotide complex and the relatively modest (twofold) change in K_D for AMP-PNP. The subsequent increase in K_D for AMP-PNP, as the $MgCl_2$ concentration is increased to the millimolar range, suggests an effect of Mg^{2+} through the low-affinity substrate sites. The concentration of Mg^{2+} for its half-maximal effect is approximately 1 mM, consistent with estimates of free Mg^{2+} binding to the enzyme [19, 20] and with the concentration dependence for Mg^{2+} as a modifier of such processes as the ADP/ATP exchange reaction [21, 22], the $K_{0.5}$ for Na^+ as activator of enzyme phosphorylation and of the Na^+-ATPase reaction [23], and of activation of the K^+-phosphatase reaction [24, 25], all these reactions being catalyzed by this enzyme [1–3].

Monovalent cations also affect the binding of AMP-PNP. K^+ decreased the binding in the presence or absence of $MgCl_2$, just as in kinetic studies K^+ increased the K_m for ATP and in binding studies (in the absence of $MgCl_2$) increased the K_D for ATP [8, 9, 14, 15]. By contrast, the effect of Na^+ depended on $MgCl_2$. In the absence of $MgCl_2$, Na^+ decreased the K_D and thus increased AMP-PNP binding. The Na^+-induced increment in binding was half-maximal near 0.3 mM, in good agreement with the $K_{0.5}$ for Na^+ as an activator of the enzyme [23, 26]. In the presence of $MgCl_2$, however, Na^+ increased the K_D for AMP-PNP; this sensitivity to Mg^{2+} may reflect the interactions between Na^+ and Mg^{2+} sites of the enzyme demonstrated by Grisham and Mildvan [19]. The apparent competition between Na^+ and K^+, manifested in changes in the apparent K_D for AMP-PNP here, has been demonstrated previously in terms both of substrate binding and of enzyme activation [9, 15, 26].

Finally, a substrate for the K^+-dependent phosphatase reaction, *p-*

nitrophenyl phosphate, was not a competitor toward AMP-PNP binding to the high-affinity substrate site. This observation is in accord with previous studies on the phosphatase reaction indicating no competition toward that substrate with micromolar concentration of either ATP or AMP-PNP, but apparent competition with millimolar concentration of either ATP or AMP-PNP [5]. Similarly, in kinetic studies of the $(Na^+ + K^+)$-ATPase reaction, p-nitrophenyl phosphate acted as a competitor to ATP at the low-affinity substrate sites [5]. Thus occupancy of the low-affinity substrate site by p-nitrophenyl phosphate would seem to have little effect on nucleotide binding to the high-affinity sites even though Mg^{2+} at concentrations sufficient to occupy the low-affinity substrate sites did increase the K_D for AMP-PNP substantially. A similar disparity has been noted in the case of the Na^+-ATPase reaction, where Mg^{2+} at the low-affinity substrate sites increased the $K_{0.5}$ for Na^+ although the Mg-ATP complex at concentrations sufficient to occupy those sites had no such effect [23].

Acknowledgment

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