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Pentavalent Ions Dependency Is a Conserved Property of Adenosine Kinase from Diverse Sources: Identification of a Novel Motif Implicated in Phosphate and Magnesium Ion Binding and Substrate Inhibition†

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ABSTRACT: The catalytic activity of adenosine kinase (AK) from mammalian sources has previously been shown to exhibit a marked dependency upon the presence of pentavalent ions (PVI), such as phosphate (PO_4), arsenate, or vanadate. We now show that the activity of AK from diverse sources, including plant, yeast, and protist species, is also markedly enhanced in the presence of PVI. In all cases, PO_4 or other PVI exerted their effects primarily by decreasing the K_m for adenosine and alleviating the inhibition caused by high concentrations of substrates. These results provide evidence that PVI dependency is a conserved property of AK and perhaps of the PfkB family of carbohydrate kinases which includes AK. On the basis of sequence alignments, we have identified a conserved motif NXXE within the PfkB family. The N and E of this motif make close contacts with Mg^{2+} and PO_4 ions in the crystal structures of AK and bacterial ribokinase (another PfkB member which shows PVI dependency), implicating these residues in their binding. Site-directed mutagenesis of these residues in Chinese hamster AK have resulted in active proteins with greatly altered phosphate stimulation and substrate inhibition characteristics. The N239Q mutation leads to the formation of an active protein whose activity was not stimulated by PO_4 or inhibited by high concentrations of adenosine or ATP. The activity of the E242D mutant protein was also not significantly altered in the presence of phosphate. Although PO_4 had no effect on the $K_m^{Adenosine}$ for this mutant, the K_m^{ATP}, $K_i^{Adenosine}$, and K_i^{ATP} were significantly decreased. In contrast to these mutations, N239L or E242L mutant proteins showed greatly decreased activity with an altered Mg^{2+} requirement. These observations support the view that N239 and E242 play an important role in the binding of PO_4 and Mg^{2+} ions required for the catalytic activity of adenosine kinase.

The enzyme adenosine kinase (AK)[1] is a purine salvage enzyme which catalyses the phosphorylation of the 5′-hydroxyl of adenosine via the γ-phosphate of cosubstrate Mg/ATP^{2-}. AK is widely distributed among plants, eukaryotic microorganisms, and mammalian tissues (*1*−*25*). An important regulatory feature of AK is its potent inhibition by high concentrations of adenosine. Studies have shown that, during myocardial and cerebral ischemia, there are marked increases in the local concentration of adenosine (*26*, *27*) correlated with a decrease in pH to as low as 6.0 (*28*−*31*). Elevated levels of adenosine have been associated with both cardiac (*26*, *32*) and neural protection (*27*, *33*), and AK is indicated to be the primary enzyme involved in its regulation under both physiological and ischemic conditions (*7*, *31*).

The mechanism of AK activity reportedly varies among enzyme sources, though the majority of studies on mammalian enzymes suggest a sequential mechanism (*34*−*38*). However, these studies report differing orders of substrate binding and product release. Difficulties in the interpretation of AK kinetic data giving rise to conflicting results are, in part, due to inhibition of AK activity by high concentrations of adenosine, excess free magnesium (Mg^{2+}), and excess ATP^{4-}. Widely different assay conditions have been reported, revealing an intricate relationship between pH and initial velocity. It is well-documented that the optimum pH for activity is dependent on the ratio of ATP/Mg^{2+} (*10*, *34*, *39*−*41*). Optimum activity is also dependent on the ratio of Mg^{2+}/adenosine (*10*, *41*, *42*). A depression in pH, as that which physiologically occurs during ischemia, thus can have a marked effect on the activity of AK.

AK belongs to the PfkB family of carbohydrate kinases, classified on the basis of two unique sequence motifs. Members of this family include ribokinase (RK:EC 2.7.1.15), inosine-guanosine kinase (EC2.1.7.73), fructokinase (EC 2.7.1.4), and 1-phosphofructokinase (EC 2.1.7.56) (*43*, *44*). The three-dimensional (3D) structure of two PfkB family members, AK and RK, have been solved and are found to be remarkably similar (*45*−*48*). The structure of bacterial

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[1] Abbreviations: ado, adenosine; AK, adenosine kinase; AsO_4, arsenate; CHO, Chinese hamster ovary; Mg^{2+}, free magnesium; MO_6, magnesium coordinated to six water molecules; PVI, pentavalent ions; PO_4, phosphate; RK, ribokinase; VO_4, vanadate.

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RK was resolved with ribose, ADP, and a bound phosphate near the active site (*47*). The distance of the phosphate ion from the γ-phosphate of ADP suggests that this phosphate does not represent the γ-phosphate of ATP. This phosphate ion makes close contacts with conserved asparagine and glutamic acid residues at sequence positions 187 and 190, respectively (Figure 1B). The corresponding residues (positions 223 and 226 in the human sequence and positions 239 and 242 in Chinese hamster sequence) are conserved in AK and occupy similar 3D space (Figure 1A). The asparagine and glutamic acid residues at these positions are also conserved in other proteins belonging to the PfkB family of carbohydrate kinases, thus identifying a new sequence motif NXXE, which is a common characteristic of these proteins (Figure 2).

The human AK protein was crystallized in the presence of adenosine and magnesium. The 3D structure shows two adenosine molecules and a magnesium ion, which has octahedral coordination to six ordered waters (MO_6) bound at the active site (*45*). Five of these six ordered waters make H-bond interactions with the protein, which include side-chain contacts with conserved NXXE residues asparagine 223 and glutamic acid 226 as well as with the putative catalytic base, aspartic acid 300 (Figure 1A). MO_6 interacts with O5′ of adenosine by a close contact of 3.52 Å as well as 2 H-bonds through a bridging water molecule. The position of MO_6 differs from that of Mg^{2+} found in the active site of *Toxoplasma gondii* AK (Figure 1C), which is coordinated between the α- and β-phosphates of AMP−PCP (a nonhydrolyzable ATP analogue), though forms only one hydrogen bond with a nonbridging oxygen of the β-phosphate (*46*). This Mg^{2+} does not show typical octahedral coordination as is shown for MO_6. For clarity, we have designated Mg^{2+} which is coordinated to AMP−PCP as M1 and the magnesium species MO_6 which is located near the 5′-hydroxyl of adenosine as M2.

We have shown that the activity of mammalian AK as well as bacterial RK is greatly affected by pentavalent ions (PVI), such as phosphate, arsenate, and vanadate. The maximum velocity increased and K_m for the phosphate-accepting substrate were decreased in the presence of PVI at physiological pH (*49*−*52*). At more acidic pH values, which mimic conditions of ischemia (*49*, *50*), AK activity showed an absolute requirement for the presence of PVI. To determine if this effect is conserved among species, we have examined the effect of PVI on the activity of AK from Chinese hamster ovary (CHO) cells, spinach, *Saccharomyces cerevisiae*, and *Leishmania donovani*. Our results show that the PVI dependency, or stimulation of AK activity by these ions, is a conserved property of AK from various sources.

The catalytic activities of both AK as well as bacterial RK are also inhibited by high concentrations of Mg^{2+}. Because both PO_4 and Mg^{2+} interact with the asparagine and glutamic acid residues of the NXXE motif, the possibility that this motif may be involved in their binding and catalytic effect was studied. Our studies show that the mutational alterations of Asn239 and Glu242 in CHO AK sequence resulted in proteins with greatly altered PO_4 effect and increased tolerance for Mg^{2+}, providing evidence that these residues are involved in PO_4 and Mg^{2+} binding and that they affect AK activity.

EXPERIMENTAL PROCEDURES

Cell Extracts. Wild-type (WT) CHO cells grown in α-MEM media supplemented with 5% fetal bovine serum were harvested near confluency, pelleted, and washed (*53*). The cell pellet was suspended in a 50 mM Tris-maleate buffer (pH 7.4) and 100 mM NaCl, sonicated to break open the cells, and then centrifuged to remove cellular debris. The cell lysate was stored at −70 °C after supplementing it with 5% glycerol and 0.1 mM DTT and EDTA (final concentrations). Yeast DL-1 *S. cerevisiae* was grown in YPD media supplemented with 20% dextrose for 48 h. The pellet from a 10 mL culture was resuspended in a 50 mM Tris-maleate buffer (pH 7.4) containing 100 mM NaCl. The suspension was vortexed with 425−600 μm glass beads for 6 × 30 s bursts, then centrifuged to remove cellular debris. The cell extract was stored at −70 °C after the addition of 5% glycerol and 0.1 mM of DTT and EDTA.

Spinach leaf extract (*Spinacia oleracea*) was obtained from Dr. Elizabeth Weretilnyk and Dr. Peter Summers from the Department of Biology at McMaster University, Hamilton (Ontario, Canada). The cell extracts from these leaves in the aforementioned buffer were made as described in Weretilnyk et al. (*54*). *L. donovani* cells were kindly provided to us by Dr. Neil Reiner, Department of Medicine, University of British Columbia, Vancouver (Canada). The cells were sonicated in a 50 mM Tris-maleate buffer (pH 7.4) and 100 mM NaCl. The cell suspension was centrifuged, and the extract was stored as described previously.

Site-Directed Mutants of Adenosine Kinase. The cloning of full length CHO AK cDNA has been described in our earlier work (*21*). The AK cDNA was subcloned into pET-15b (Novagen) expression vector with a $(His)_6$ tag at the N-terminal end, as previously described (*55*). Site-directed mutagenesis at N239 and E242 was made by the use of "Quikchange" site-directed mutagenesis kit (Stratagene), as described in earlier work (*55*). The changes in the mutants N239Q, N239L, E242D, and E242L were verified by DNA sequencing. After the transformation of BL21(D3) cells with the mutant plasmids, expression of the recombinant proteins was induced by the addition of 0.1 mM IPTG. The recombinant AK proteins from the WT and different mutant clones were purified by nickel affinity chromatography, as previously described (*49*).

Adenosine Kinase Activity Assays. The AK activity was measured by means of a radioactive assay involving the conversion of ^{3}H-adenosine into ^{3}H-AMP, as described earlier in our work (*49*, *51*, *55*). The standard reaction mixture for AK activity determination contained a 50 mM Tris-maleate buffer, 10 μM ^{3}H-adenosine (specific activity = 265 mCi/mmol), 1 mM ATP, and 1.78 mM $MgCl_2$. The assays were carried out at either pH 7.4 (cell extracts and recombinant proteins) or at pH 6.2 (purified recombinant proteins). The lower pH was used to mimic the ischemic conditions. (2,8-^{3}H)-adenosine (30.1 Ci/mmol) was obtained from DuPont Canada Inc. All other chemicals were of analytical reagent grade. Reactions were initiated by the addition of either 12 μg protein from cell extracts or 47 pmol purified AK to a final 300 μL volume of reaction mixture at 37 °C. Initial velocities were determined by withdrawing 50 μL samples at various time intervals and by adding these to 1 mL of cold $LaCl_2$ in order to precipitate the radio-labeled

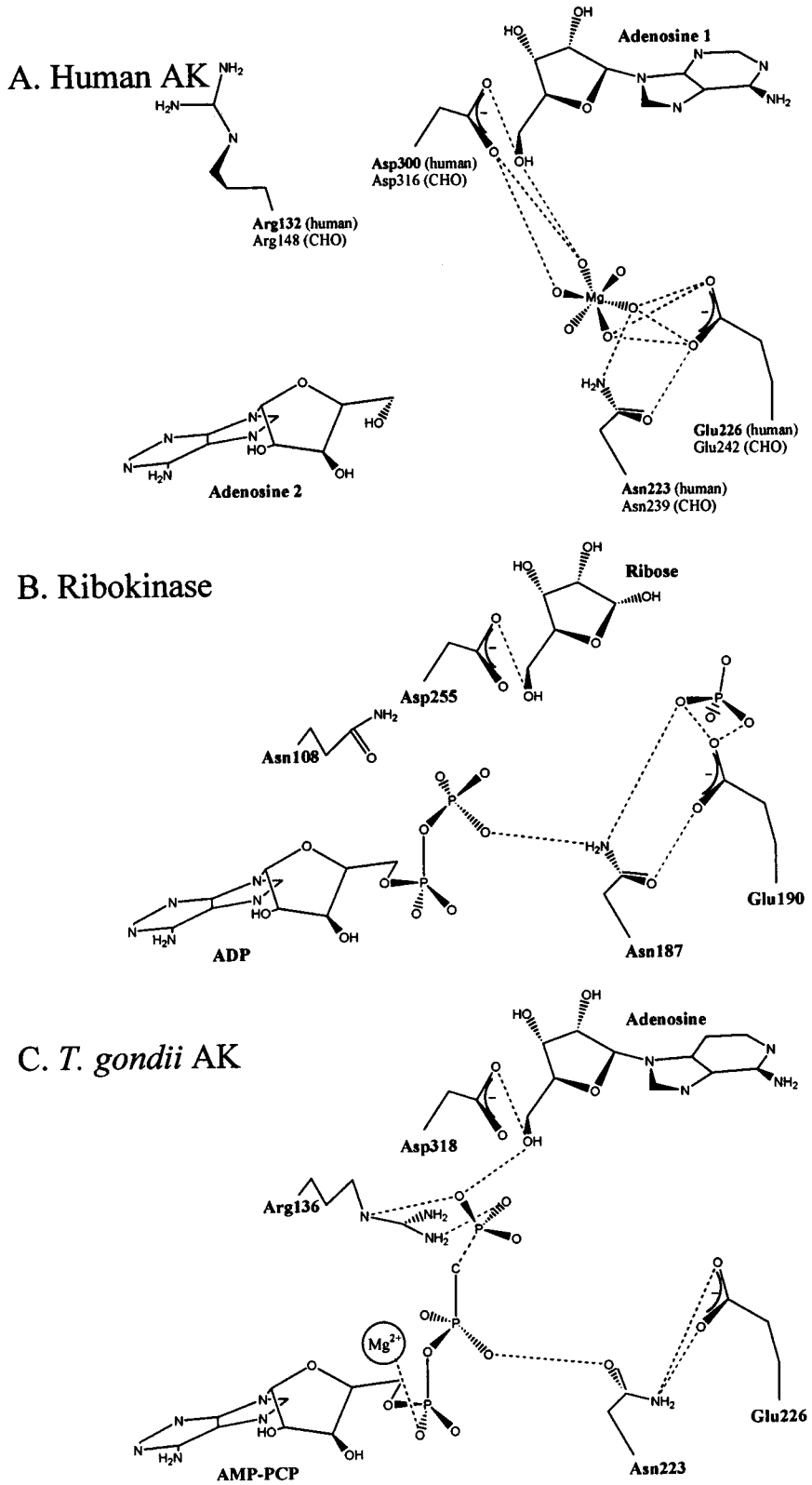

FIGURE 1: Active site structure of adenosine kinase and ribokinase. The hydrogen-bond network between residues and active site ligands are shown. The putative catalytic base, aspartic acid, and NXXE residues asparagine and glutamic acid are absolutely conserved among AK and RK. (A) Structure of human AK active site, with the corresponding residue numbering for CHO AK. H-bonding from M2 to N239 and E242 are shown. The approximate position of R148 in its resting state is also depicted. (B) Ribokinase active site is shown with ligands ribose and ADP. The relative position of PO_4 and interactions with asparagine and glutamic acid are shown. (C) Structure of *T. Gondii* AK. An arginine residue which is absolutely conserved among AK, undergoes domain and side-chain rotation to position near the γ-phosphate of AMP−PCP. M1 is coordinated between the α- and β-phosphates of AMP−PCP.

AMP formed in the reaction. After being kept on ice for a minimum of 3 h, the precipitate was collected by filtration on a glass fiber filter and washed with 20 volumes of cold distilled water. The dried glass fiber filters were placed in 5 mL scintillation vials with aqueous scintillation fluid and the radioactivity was measured on a Beckman LS 7800

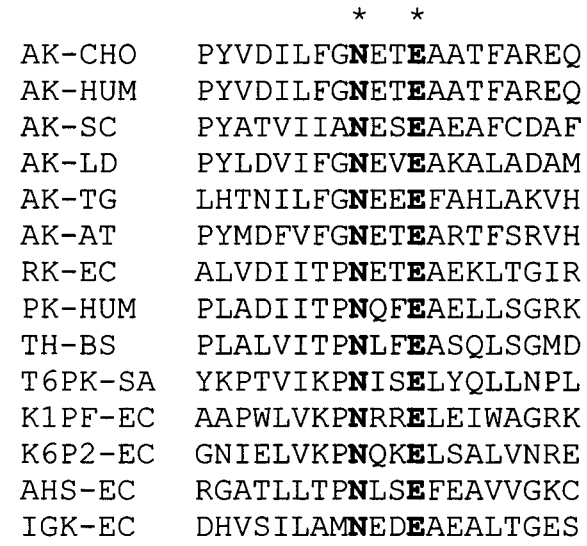

FIGURE 2: Sequence alignment of representative proteins from PfkB family of carbohydrate kinases. The conserved NXXE motif is marked with asterisks and highlighted in boldface type. The sequence identifications are as follows: AK-CHO, Chinese hamster ovary AK; AK−HUM, human AK; AK-SC, *S. cerevisiae* AK; AK-LD, *L. donovani* AK; AK-TG, *T. gondii* AK; AK-AT, *A. thaliana* AK; RK-EC, *Escherichia coli* ribokinase; PK-HUM, human pyridoxal kinase; TH-BS, *B. subtilis* phosphomethylpyrimidine kinase; T6PK-SA, *S. aureus* tagatose-6-phosphate kinase; K1PF-EC, *E. coli* 1-phosphofructokinase; K6P2-EC, *E. coli* 6-phosphofructokinase isozyme 2; AHS-EC, *E. coli* ADP heptose synthase; IGK-EC, *E. coli* inosine/guanosine kinase.

scintillator. Kinetic constants were determined by plotting the initial velocities against varying substrate concentrations using Graphpad Prism, version 1.03.

Optimum activity for the *L. donovani* protein required a concentration of 2.88 mM $MgCl_2$ and fixed adenosine concentrations of 30 μM. For $K_m^{Adenosine}$ and K_m^{ATP} determinations, the mutant enzyme N239Q required fixed concentrations of adenosine at 100 μM and ATP at 100 mM. K_m^{ATP} for the mutant enzyme E242D required a fixed concentration of 20 μM adenosine. In the absence of phosphate, the optimum concentration of ATP for E242D was 20 mM; in the presence of 20 mM PO_4, the optimum concentration was 2 mM.

Molecular Modeling. 3D coordinates for human AK (1BX4), ribokinase (1RKD), and *T. gondii* AK (1DGY) were obtained from the Protein Data Bank (*56*). The coordinates were imported into Swiss-PBD viewer, version 3.6B2, software and superimposed, and a structural alignment was performed. The RMS deviation of the backbone was then minimized. The 3D coordinates for M2 of the human AK structure and PO_4 of the ribokinase structure were then modeled into the active site of *T. gondii* AK (already containing coordinates for adenosine and AMP−PCP ligands), retaining H-bond distances and angles from residues in the original structures. No steric clashes between protein or ligands were generated. Active-site diagrams were then reconstructed into two dimensions with CS ChemDraw Pro, version 4.5.

RESULTS

We have previously shown that PVI, such as phosphate, arsenate, and vanadate, stimulated the activity and decreased the K_m of AK for adenosine from several mammalian sources (viz., Chinese hamster, Syrian hamster, beef liver, and human) (refs *49* and *51* and unpublished results). Recently, bacterial RK, which is another member of the PfkB family, was also shown to be similarly affected by PVI (*50*). To understand the role of PVI in the activity of AK, it was necessary, at first, to determine whether the requirement of PVI for activity was a common characteristic of AK from divergent sources and then to identify the residues that may be responsible for this effect.

Table 1: Steady-State Kinetic Parameters of Cosubstrate Adenosine for AK in Cell Extracts

enzyme	PVI	(mM)	K_m[a] (μM)	V_{max} (pmol s^{-1} μg^{-1})	K_i (μM)
CHO cells		0	31.8 ± 24.9	0.038 ± 0.025	9.5 ± 7.6
	PO_4	1	6.6 ± 2.2	0.077 ± 0.006	29.4 ± 4.2
		5	1.7 ± 0.2	0.102 ± 0.004	55.8 ± 7.4
		20	0.8 ± 0.1	0.108 ± 0.002	97.1 ± 9.2
	AsO_4	20	4.9 ± 3.9	0.102 ± 0.005	66.1 ± 8.8
	VO_4	20	7.8 ± 5.6	0.066 ± 0.005	48.0 ± 7.8
spinach		0	19.7 ± 7.4	0.026 ± 0.007	38.4 ± 17.3
	PO_4	1	4.6 ± 0.8	0.032 ± 0.003	76.5 ± 16.3
		5	1.3 ± 0.3	0.068 ± 0.005	81.5 ± 22.9
		20	0.9 ± 0.1	0.083 ± 0.003	123.2 ± 24.0
	AsO_4	20	2.5 ± 0.6	0.080 ± 0.007	65.2 ± 16.0
	VO_4	20	4.8 ± 1.1	0.045 ± 0.005	44.2 ± 11.2
yeast		0	25.9 ± 16.7	0.011 ± 0.006	18.4 ± 12.9
	PO_4	1	11.3 ± 2.0	0.057 ± 0.006	165.3 ± 53.2
		5	6.0 ± 0.7	0.114 ± 0.006	483.2 ± 188.3
		20	5.5 ± 0.8	0.123 ± 0.008	444.1 ± 200.3
	AsO_4	20	11.2 ± 0.8	0.129 ± 0.005	242.4 ± 41.6
	VO_4	20	11.7 ± 1.7	0.106 ± 0.009	63.6 ± 11.9
L. donovan		0	12.5 ± 1.8	0.003 ± 0.0001	*b*
	PO_4	0.1	3.0 ± 0.4	0.006 ± 0.0002	*b*
		1	1.7 ± 0.1	0.023 ± 0.0003	*b*
		5	0.8 ± 0.05	0.030 ± 0.0003	*b*
		20	0.6 ± 0.1	0.034 ± 0.001	*b*
	Ars	20	4.3 ± 0.5	0.035 ± 0.002	222.7 ± 50.9
	VO_4	20	6.7 ± 1.9	0.032 ± 0.001	56.84 ± 19.7

[a] The K_m in cell extracts should be considered as apparent values. [b] Substrate inhibition not detected.

Effects of Phosphate on the Initial Velocity of AK from Diverse Sources. We have examined the effect of increasing PVI concentration on the activity of AK in cell extracts from plant, yeast, CHO, and the parasitic organism *L. donovani*. Tables 1 and 2 show the effects of different concentrations of PVI on the kinetic parameters of AK from these sources. The PVI increased the maximum velocity in all cases, which was correlated with a substantial increase in K_i and decrease in K_m for adenosine. Because both adenosine and ATP are also metabolized to certain extent by other enzymes present in cell extracts, the K_m values observed under such conditions should be regarded as apparent values. The results obtained in these studies are discussed in the following section.

Similar to our earlier studies with purified AK from Syrian hamster and beef liver (*49*, *51*, *52*) and recombinant CHO (*49*), the activity of AK from CHO cell extracts was stimulated in the presence of PVI. The K_i for adenosine increases from 9.5 μM in the absence of PVI to 97.1, 66.1, and 48.0 μM in the presence of 20 mM phosphate, arsenate, and vanadate, respectively. As shown for PO_4, the decrease in K_i occurred in a concentration-dependent manner. The maximum velocity increases and $K_m^{Adenosine}$ decreases upon the addition of increasing concentrations of PO_4 and in the presence of 20 mM arsenate and vanadate (Table 1). The

Table 2: Steady-State Kinetic Parameters of Cosubstrate ATP for AK in Cell Extracts

enzyme	PVI	(mM)	K_m[a] (mM)	V_{max} (pmol s^{-1} μg^{-1})
CHO cells		0	*b*	*b*
	PO_4	20	0.142 ± 0.010	0.114 ± 0.142
	AsO_4	20	0.151 ± 0.022	0.059 ± 0.151
	VO_4	20	0.149 ± 0.016	0.035 ± 0.149
spinach		0	0.105 ± 0.014	0.012 ± 0.105
	PO_4	20	0.105 ± 0.004	0.072 ± 0.105
	AsO_4	20	0.109 ± 0.012	0.062 ± 0.109
	VO_4	20	0.101 ± 0.006	0.028 ± 0.101
yeast		0	0.060 ± 0.005	0.004 ± 0.060
	PO_4	20	0.060 ± 0.008	0.092 ± 0.060
	AsO_4	20	0.060 ± 0.005	0.070 ± 0.060
	VO_4	20	0.057 ± 0.007	0.053 ± 0.057
L. donovan		0	*b*	*b*
	PO_4	20	0.049 ± 0.006	0.034 ± 0.049
	AsO_4	20	0.052 ± 0.004	0.032 ± 0.052
	VO_4	20	0.050 ± 0.003	0.026 ± 0.050

[a] The K_m in cell extracts should be considered as apparent values. [b] Activity too low to calculate constants.

apparent K_m for ATP was not affected in the presence of PVI and remained at approximately 0.15 mM under all conditions (Table 2). The effects of PVI on the spinach enzyme were very similar to the mammalian enzyme on the kinetic constants of $K_i^{Adenosine}$, maximum velocity, and $K_m^{Adenosine}$ (Table 1). The K_m^{ATP} for this enzyme also was not altered in the presence of PVI (Table 2).

The maximum velocity of the yeast (*S. cerevisiae*) AK was markedly increased upon the addition of PVI (Table 1). The $K_i^{Adenosine}$ for yeast AK is similar to the mammalian enzyme in the PVI-free form, but in the presence of 20 mM phosphate and arsenate, the concentration of adenosine necessary to invoke substrate inhibition was approximately 4-fold higher in comparison to the mammalian and plant AK. The apparent K_m of the enzyme for ATP remained constant under all conditions and approximately 2-fold lower than that of the CHO enzyme (Table 2).

The AK from *L. donovani* AK, as reported in the literature (*41*), was not inhibited by excess Mg^{2+} or high concentrations of adenosine. However, similar to AK from other sources, the addition of PO_4 as well as other PVI lead to a stimulation of its activity and a reduction in the K_m for adenosine (Table 1). In the presence of 20 mM arsenate and vanadate, some inhibition of enzyme activity was seen at high adenosine concentrations. The apparent K_m of the enzyme for ATP remained constant at approximately 0.050 mM under all conditions (Table 2).

Effects of Phosphate on Site-Directed Mutants Altered in the NXXE Motif. Our studies of the AK and RK structures indicate that the N239 and E242 residues in CHO AK, which are part of the conserved NXXE motif, interact with PO_4 and Mg^{2+} ions. To determine the importance of these interactions for the activity of AK, we have made specific alterations in these residues of CHO cDNA by site-directed mutagenesis. The mutants that we have generated consisted of replacing N239 with either glutamine (N239Q) or leucine (N239L) and replacing Glu242 with either aspartic acid (E242D) and leucine (E242L). The recombinant WT and the mutant AK proteins were purified, and their responses to PO_4 and Mg^{2+} were studied. Preliminary studies with these enzymes at both pH 7.4 and 6.2 showed similar effects.

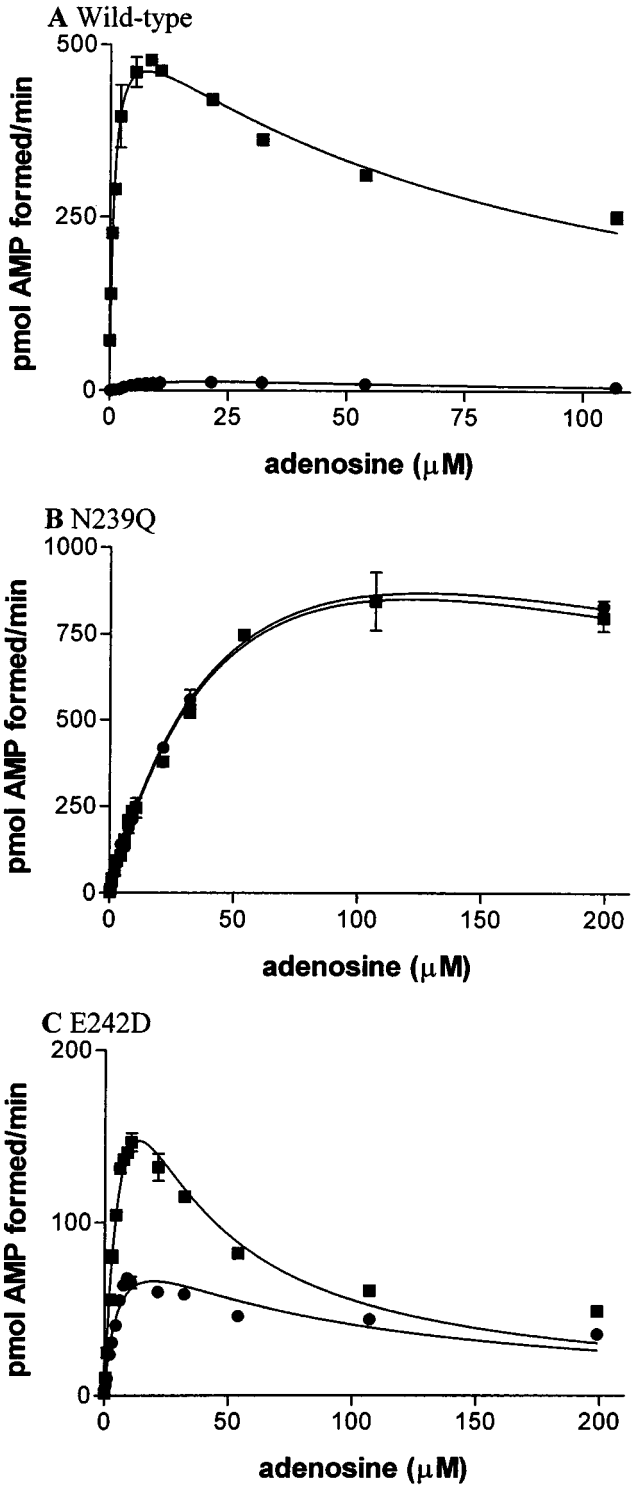

FIGURE 3: Effects of pentavalent ions on the initial velocity and K_m for adenosine. Initial velocity is plotted against adenosine concentrations for WT and mutant AK: (A) WT in the absence (●) and in the presence of 20 mM phosphate (■); (B) N239Q in the absence (●) and presence of 20 mM phosphate (■); (C) E242D in the absence (●) and presence of 20 mM phosphate (■).

However, the magnitude of the effects was higher at pH 6.2 than at pH 7.4 (results not shown). Therefore, further studies with these proteins were carried out at pH 6.2.

Figures 3 and 4 show the secondary plots of initial velocity versus substrate concentration for WT AK and two of the mutants, N239Q and E242D. The other two mutants, N239L and E242L, showed negligible AK activity under the standard

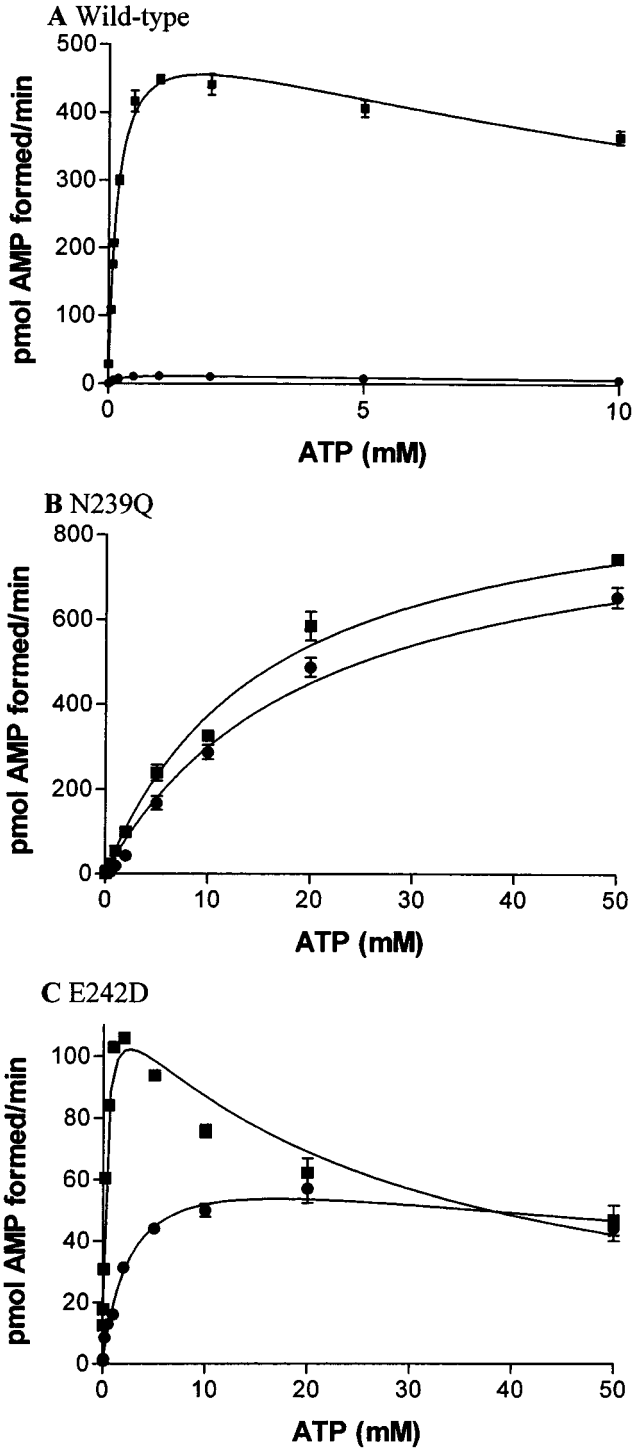

FIGURE 4: Effects of pentavalent ions on the initial velocity and K_m for ATP. Initial velocity is plotted against ATP concentrations for WT and mutant AK: (A) WT in the absence of pentavalent ions (●) and in the presence of 20 mM phosphate (■); (B) N239Q in the absence (●) and presence of 20 mM phosphate (■); (C) E242D in the absence (●) and presence of 20 mM phosphate (■).

assay conditions, and their results will be discussed later. As seen from Figures 3 and 4, WT AK shows negligible activity in the absence of PO_4, and activity is greatly stimulated upon the addition of PO_4. In contrast, the N239Q mutation results in a protein which shows substantial activity in the absence of PO_4, and there is no appreciable change in activity upon the addition of PO_4 (Figures 3B and 4B). Further, in contrast to the WT enzyme which showed

Table 3: Steady-State Kinetic Parameters of Cosubstrate Adenosine for Wild-Type and Site-Directed Mutants

enzyme	PVI (mM)		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	K_i (μM)
wild-type	PO_4	0	18.8 ± 7.7	0.08 ± 0.02	4.16×10^3	22.2 ± 9.9
		20	0.8 ± 0.1	1.18 ± 0.04	1.47×10^6	76.0 ± 9.4
N239Q	PO_4	0	64.5 ± 6.4	3.73 ± 0.26	5.78×10^4	249.1 ± 42.0
		20	69.1 ± 12.7	3.85 ± 0.51	5.57×10^4	217.8 ± 64.2
E242D	PO_4	0	6.0 ± 2.1	0.23 ± 0.04	3.79×10^4	66.2 ± 26.0
		20	10.8 ± 3.5	0.80 ± 0.20	7.42×10^4	17.6 ± 5.7

Table 4: Steady-State Kinetic Parameters of Cosubstrate ATP for Wild-Type and Site-Directed Mutants

enzyme	PVI (mM)		m (mM)	k_{cat} (s^{-1})	K_i (mM)
wild-type		0	0.17 ± 0.03	0.03 ± 0.01	9.6 ± 2.2
	PO_4	20	0.16 ± 0.01	1.14 ± 0.03	20.3 ± 2.1
N239Q		0	31.15 ± 7.56	2.57 ± 0.41	280.8 ± 168.3
	PO_4	20	21.82 ± 4.33	2.50 ± 0.30	421.0 ± 266.1
E242D		0	2.68 ± 0.47	0.15 ± 0.01	109.3 ± 25.5
	PO_4	20	0.25 ± 0.07	0.26 ± 0.02	26.4 ± 8.0

inhibition at higher concentrations of substrates, the activity from N239Q mutant was not inhibited at high concentrations of either adenosine or ATP. The mutant protein E242D also showed interesting changes in its response to PO_4. This protein also exhibited substantial AK activity in the absence of PO_4 and was stimulated only about 3-fold in the presence of 20 mM PO_4. The effects of these mutations on the properties of AK can be best understood by the values of various kinetic constants for the WT and the mutant enzymes (Tables 3 and 4).

As seen from Table 3, the $K_m^{Adenosine}$ for the N239Q enzyme in the absence of phosphate is approximately 3.5-fold higher than that of the WT enzyme. However, in contrast to the WT, where $K_m^{Adenosine}$ is greatly reduced in the presence of PO_4 (more than 20-fold), the K_m for the mutant enzyme was not significantly affected by phosphate. As a result, the k_{cat}/K_m ratio for the N239Q protein is about 14-fold higher than that for WT in the absence of PO_4. However, in the presence of PO_4, the k_{cat}/K_m ratio of the mutant protein is about 20-fold lower than that of WT. Another significant change seen for the N239Q protein was that the K_m for ATP is about 200-fold higher than seen for WT AK. The K_m^{ATP} for the mutant protein was slightly reduced in the presence of PO_4 (Table 4).

The E242D protein was less active than the WT protein. The k_{cat} for this protein is increased about 2−3-fold in the presence of phosphate (Table 3). The K_m for adenosine is higher and $K_i^{Adenosine}$ is also somewhat lower in the phosphate-bound form (Table 3). The most interesting effect of this mutation is seen in the K_m for ATP (Table 4), which is found to be about 15-fold higher in the absence of phosphate and then reduced by a factor of 10 in the presence of phosphate. This is the only enzyme for which the K_m for ATP is significantly altered in the presence of PO_4 (Table 4). Another interesting feature of this mutant is that K_i^{ATP} is decreased approximately 3-fold in the presence of phosphate, to a value similar to that seen for WT AK under similar conditions (Table 4).

Mg^{2+} Ion Dependency of the WT AK and Various Mutants Affected in the NXXE Motif. We have examined the activity of WT CHO AK as a function of free Mg^{2+} in the presence

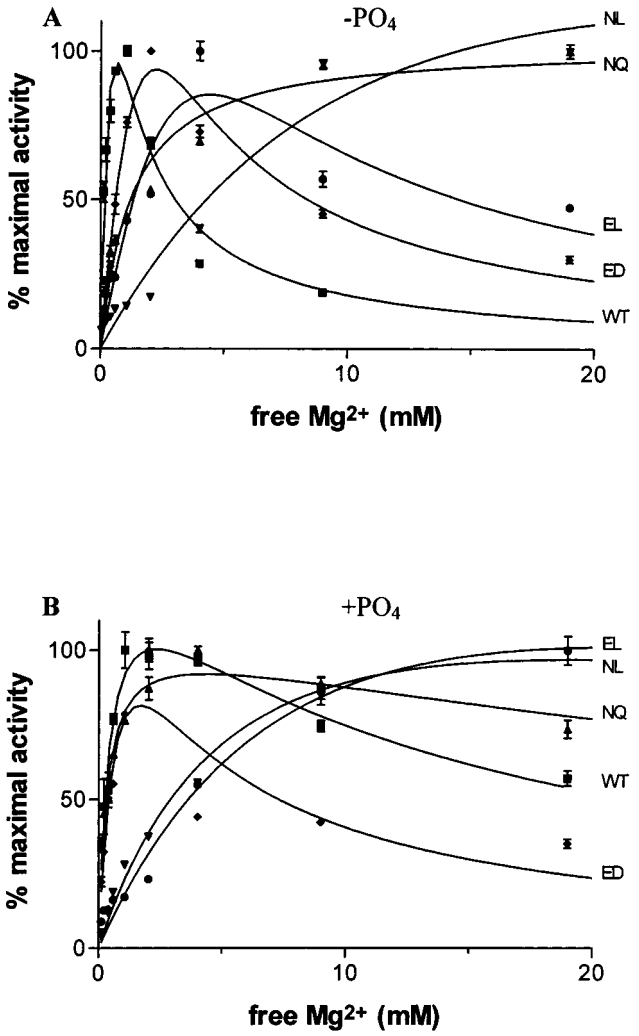

FIGURE 5: Magnesium ion dependencies of WT and various mutant AK. Percent maximal velocities are plotted against free magnesium concentrations in the absence (A) and presence (B) of 20 mM phosphate for WT (■), N239L (▼), N239Q (▲), E242L (●), and E242D (♦). Kinetic parameters determined from curve fitting are reported in Table 3.

and absence of phosphate so that any changes in the mutants could be determined. The calculation of free Mg^{2+} was performed as described by Fabiato and Fabiato (*57*). In solution at pH 6.2 and at 1 mM ATP and 0.5 mM $MgCl_2$ (0.15 mM free Mg^{2+}), ATP is complexed to nearly 35% by magnesium. Under these conditions, there was no detectable WT activity. Above 0.75 mM $MgCl_2$ (0.26 mM free Mg^{2+}), the initial velocity of WT increases hyperbolically to maximal at 1 mM free Mg^{2+} (Figure 5). Above concentrations of 1 mM, free Mg^{2+} increasingly inhibits activity. In the presence of 20 mM phosphate, the apparent K_{act}^{Mg2+} for WT AK is not altered (0.4 mM); however, the apparent K_i^{Mg2+} is increased by about 15-fold (Table 5).

The initial velocity of various mutants was examined as a function of free Mg^{2+}. The magnitude of substrate inhibition by adenosine, and to a lesser extent Mg^{2+}/ATP, are influenced by the concentration of free Mg^{2+} (*10*, *58*), all of which are inter-related with the pH of the assay (*8*). It should be noted that these experiments were carried out at fixed substrate concentrations (10 μM adenosine and 1 mM ATP) which were optimal for the WT enzyme at 1 mM free Mg^{2+}. As a result, the derived kinetic constants for the WT and mutant enzymes are likely suboptimal and should be considered as apparent values, included for comparative purposes only.

Table 5: Steady-State Kinetic Parameters of Mg^{2+} for Wild-Type and Mutant AK

enzyme	PO_4 (mM)	K_{act} (mM)	k_{cat} (s^{-1})	K_i (mM)
wild-type	0	0.6 ± 0.5	0.068 ± 0.036	0.7 ± 0.5
	20	0.4 ± 0.1	0.159 ± 0.016	12.4 ± 3.5
N239Q	0	1.3 ± 0.2	0.016 ± 0.001	[a]
	20	0.3 ± 0.1	0.146 ± 0.008	54.9 ± 13.9
N239L	0	15.5 ± 10.0	0.001 ± 0.0004	53.2 ± 50.1
	20	4.4 ± 0.6	0.008 ± 0.001	493.4 ± 424.4
E242D	0	8.3 ± 16.3	0.082 ± 0.148	0.6 ± 1.2
	20	0.8 ± 0.7	0.171 ± 0.089	3.5 ± 3.1
E242L	0	14.0 ± 39.5	0.0002 ± 0.001	1.4 ± 4.0
	20	11.0 ± 4.7	0.003 ± 0.001	41.4 ± 23.4

[a] Substrate inhibition not detected.

N239 makes one close contact with M2 (3.5 Å) in the structure of the human enzyme and one close contact with O3 of phosphate (4.0 Å) in the RK structure (Figure 1, A and B). This residue also makes close contacts with a nonbridging oxygen of the β-phosphate of ADP (4.3 Å) and ACP (3.5 Å) in the structure of RK and *T. gondii* AK, respectively (Figure 1, B and C). For the N239Q mutant protein, the k_{cat}^{Mg2+} and K_{act}^{Mg2+} values in the presence of PO_4 were very similar to those for the WT AK. However, in the absence of PO_4, N239Q has a k_{cat}^{Mg2+} value 4 times lower and a K_{act}^{Mg2+} value slightly higher than those for WT. Under these conditions, this enzyme is not inhibited by high concentrations of Mg^{2+} (Table 5). For the E242D mutant, the K_{act}^{Mg2+} is higher in the absence of phosphate but is decreased to a value comparable to that of the WT upon the addition of PO_4. Both the N239L and E242L mutants displayed only weak activity, even at very high concentrations of Mg^{2+}. Hence, for clarity sake, the activity data for these mutants are presented by percent maximal activity for each protein studied. Because of the low activity seen with these mutants, the errors in various constants are high for these proteins (Table 5). However, for both of these mutants, the K_{act}^{Mg2+} was found to be between 10- and 25-fold higher than that for the WT enzyme, both in the presence and absence of PO_4, and the activity was significantly enhanced by phosphate.

DISCUSSION

Adenosine kinase is a key enzyme in the metabolism of adenosine, which shows potent pharmacological activity (*33*, *59*−*61*). Although AK has been studied for more than 30 years, its mechanism of action is still not completely understood, and novel and important regulatory features of this enzyme continue to be uncovered. One unexpected feature of AK function discovered in our work a few years ago was that its catalytic activity required the presence of PVI, such as phosphate, arsenate, or vanadate. These studies also provided evidence that the PVI were not consumed or did not directly participate in the reaction catalyzed by AK. All previous studies on the effect of PVI on AK have been carried out with the enzyme derived from mammalian sources (*49*, *51*). Thus, an important question was whether the PVI

dependency of AK was restricted to the mammalian enzyme or whether it constituted a conserved intrinsic property of the enzyme. Results presented here now show that the pentavalent ions caused a marked enhancement in the activity of AK from various sources, including mammals, plants, yeast, and the protist species *L. donovani*. In all cases, phosphate and other PVI have been found to exert their effects primarily by decreasing the K_m for adenosine and by alleviating the inhibition caused by high concentrations of the cosubstrates. The AK from *L. donovani*, which is not inhibited at high adenosine and magnesium concentrations, is also similarly stimulated by PVI, providing evidence that the pentavalent ion dependency is an intrinsic characteristic of the enzyme from various sources. A number of cations and anions, such K^+, Na^+, Fe^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, acetate, sulfate, sulfite, nitrate, and carbonate, have previously been investigated; however, only pentavalent ions have been found to stimulate AK activity (*51*, *52*).

AK is a member of the PfkB family of carbohydrate kinases. We have recently shown that, similar to AK, the catalytic activity of RK, another member of the PfkB family, was also strongly stimulated by PVI, which acted by lowering the K_m of the enzyme for D-ribose. In addition to AK and RK, three other enzymes involved in the transfer of phosphate from ATP to a sugar derivative (viz., 6-phosphofructo-2-kinase (EC 2.7.1.105) (*62*), phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) (*63*), and 6-phosphofructokinase (EC 2.7.1.11) (*64*)) have been reported to show a similar dependence on PO_4 for activity. It is thus likely that PVI dependency is a common characteristic of the PfkB family of proteins as well as for some other carbohydrate kinases.

The mechanism by which PVI stimulates or are required for the activity of AK and RK (or the other aforementioned enzymes) is presently not understood. However, potentially useful information in this regard was provided by the crystallographic data of AK and RK. These data indicated that asparagine 239 and glutamic acid 242 in the CHO AK sequence, which are part of the conserved NXXE motif found in both these enzymes as well as various other PfkB family members, make close contacts with Mg^{2+} (M2) and PO_4 ions in their respective structures. To determine whether these residues indeed play an important role in the interaction of PO_4 and Mg^{2+} ions, these residues were replaced with other amino acids, and the effect of these alterations on the activities of these enzymes and their response to PO_4 and Mg^{2+} ions was determined. Results of these studies strongly support the contention that the NXXE motif identified by us plays an important role in the effects of these ions on the catalytic activity of AK.

Of the two conserved amino acid residues that are part of the NXXE motif, the asparagine at position 239 has an uncharged polar side chain. It was mutated to either similarly charged glutamine, which is one carbon unit longer, or to the nonpolar amino acid leucine, which is similar in size to asparagine. N239 makes close contacts with a nonbridging oxygen of the β-phosphate of ADP (4.3 Å) and AMP−PCP (3.5 Å) in the structure of RK and *T. gondii* AK, respectively (Figure 1, B and C). If N239 contributed to catalysis solely by assisting in the binding of ATP, then it is reasonable to assume that the mutation of it would mainly affect the K_m for ATP. However, the N239Q mutant showed dramatically higher K_m values for both adenosine and ATP (particularly in the presence of PO_4), and the stimulatory effect of phosphate on the catalytic activity of the enzyme was entirely eliminated. The N239L mutant showed very weak and inefficient AK activity; however, it required much higher concentrations of Mg^{2+} for optimal activity. The side chain of N239 makes one close contact with M2 (3.5 Å) in the human AK structure and a close contact with O3 of PO_4 (4.0 Å) in the RK structure (Figure 1, A and B). The replacement of this residue with the nonpolar leucine residue, which is unable to form these contacts, was expected to result in a large decline in AK activity and altered Mg^{2+} requirement. Although the exact mechanistic effect of the N239 residue in AK activity remains uncertain, it is quite clear that changes in this residue have a major impact on both phosphate stimulation and Mg^{2+} requirement.

The glutamic acid residue at position 242 interacts with two coordinated waters of M2 (<2.8 Å) in the human AK structure and also forms two contacts with the oxygens of PO_4 (2.8 and 4.2 Å) in the structure of RK (Figure 1, A and B). The side chain of E242 also forms a hydrogen bond with the main-chain nitrogen of N239. The negatively charged residue E242 was replaced with either aspartic acid, which is one carbon unit shorter but retains the negative charge, or with leucine, which is nonpolar and similar in size to aspartic acid. E242 does not directly interact with ATP or adenosine, yet in the absence of phosphate, the E242D mutant showed an increase in K_m for both ATP and Mg^{2+} as well as higher K_i for ATP as compared to WT. However, the addition of phosphate evokes an increase in K_m and a decrease in K_i for adenosine. The presence of phosphate has the opposing effect of decreasing the K_m^{ATP} and apparent K_m^{Mg2+}. These opposing effects of the E242D mutation suggest that the role of residue E242 is complex and not limited to H bonding with N239 for the optimal binding of ATP. The E242L mutation leads to an enzyme with greatly reduced activity and much higher apparent K_m for Mg^{2+}.

The observed changes in the kinetic properties of AK upon mutation of N239 and E242 strongly suggest that these residues are involved in controlling AK activity through the binding of phosphate and magnesium ions. To account for these observations and the various ligands found at the active sites of RK and human and *T. gondi*i AK, a model as to how these residues are linked and interact is suggested (Figure 6). The construction of the model is described in the Experimental Procedures section. The residue numbering of this model is based on the amino acid sequence of CHO AK. M2 from the human AK structure and PO_4 from the RK structure were modeled into the active site of *T. gondii* AK, which contains coordinates for adenosine, AMP−PCP, and M1. The distances of M2 and PO_4 to the protein and bond angles seen in the original structures were retained, and the modeling of these ligands does not interrupt any existing H-bond interactions. Crystallographic water molecules at the active site of *T. gondii* were also retained. Four of these water molecules superimpose with the coordinated waters of M2, and three of these waters are found in similar 3D space at the active site of RK.

As seen in the 3D structure of human AK, the coordinated waters of M2 form one H-bond contact with the 5′-hydroxyl of adenosine and three to the carboxylate of the putative catalytic residue Asp316. The close contacts of M2 to N239

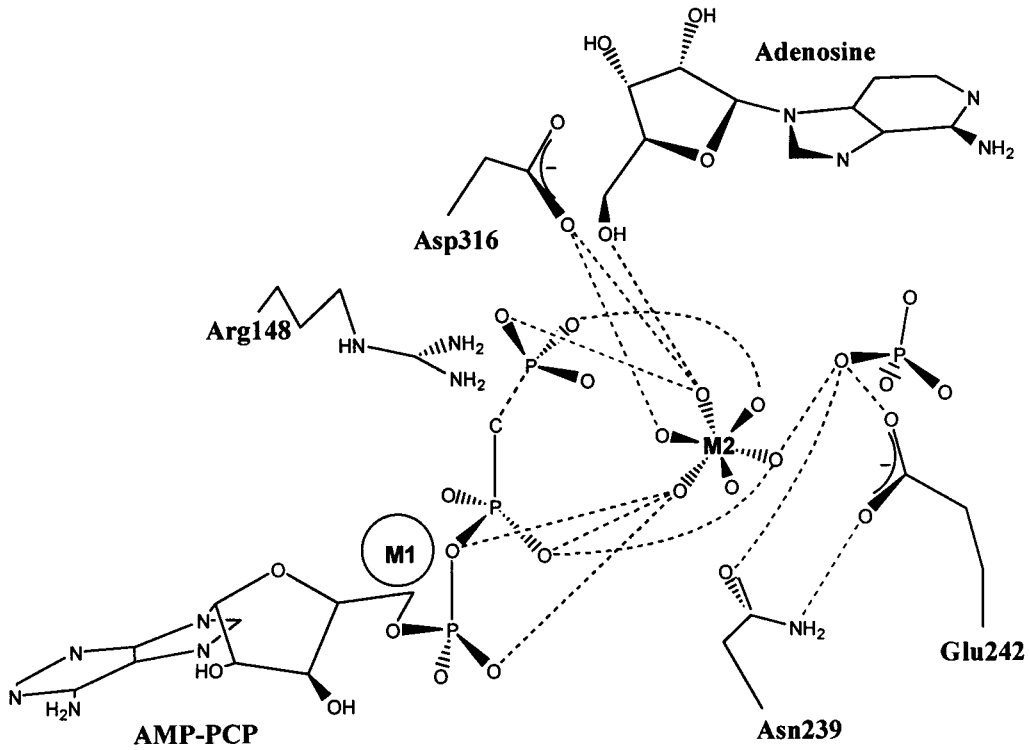

FIGURE 6: Model showing the interactions of substrates and ligands at the active site of adenosine kinase. The ligands adenosine, AMP−PCP, and M1 are found in the original *T. gondii* structure. M2 of the human AK and PO_4 of ribokinase were easily modeled into the active site of *T. gondii* with Swiss PDB Viewer, version 3.6B2. No steric clashes between existing residues or ligands were generated. The 3D image was then translated into 2D with CS ChemDraw Pro, version 4.5. The side chains of CHO AK Arg148, Asn239, Glu242, and Asp316, which form an intricate H-bond network with ligands, are absolutely conserved among AK. Two main-chain contacts from PO_4 to conserved large domain residues Ser214 and Pro216 are not shown in this substrate-bound closed conformation of AK.

and E242 are also retained. In this model, M2 forms a close contact with the bridging oxygen between α- and β-phosphates, as well as a number of H-bond contacts with nonbridging oxygens of α-, β-, and γ-phosphates of AMP−PCP. As seen in the original RK structure, the modeled phosphate retains similar distances to residues N239 and E242. In this model, PO_4 forms a hydrogen bond with a coordinated water of M2 as well as a close contact with a nonbridging oxygen of the β-phosphate of AMP−PCP (not shown).

The small domain residue arginine 148 is shown. In the original structures of *T. gondii* AK, this residue is translocated 13.7 Å toward the active site upon the binding of adenosine, then rotates into the active site upon the binding of AMP−PCP, forming two hydrogen bonds with nonbridging oxygens of the γ-phosphate (not shown). Therefore, it is thought that this residue is responsible for compensating negative charges which develop during the transfer of the phosphoryl group from ATP to adenosine (*46*). We have included this residue in our model as it forms two contacts less than 3.5 Å with the coordinated waters of M2 (not shown). PO_4 also makes three contacts with Arg148 through water molecules (not shown).

Biochemical data for AK from a variety of sources presented here and, for ribokinase (*50*), suggest that the effects of pentavalent ions are 2-fold. First, the addition of pentavalent ions evokes a reduction in K_m for the first cosubstrate to bind. A number of sequential conformational changes are required for enzyme turnover of PfkB proteins (*46*, *48*). As both substrates and products contain identical features, PVI must facilitate both the formation of the active site pocket and reduce the nonspecific binding of ligands. Second, large increases in K_i for adenosine, ATP, and Mg^{2+} for AK are seen in the presence of PVI. The present model shows that the electrophilic properties of pentavalent ions may assist in the rearrangement of charges at the active site to facilitate product release or restoring the resting state of the enzyme. M2 may be involved in the correct positioning of the arginine residue for the rearrangement of electrical charges which occur upon enzyme turnover.

The model suggests that PO_4 and M2 bound to the NXXE site are within correct distances to the active site to facilitate the productive binding of substrate and product release. Site-directed mutagenesis of the NXXE motif clearly results in enzymes with greatly altered phosphate and magnesium requirements. The model also indicates that M2, located at the NXXE motif, is within H-bonding distances to facilitate the binding of ATP, as proposed for other phosphotransferases which require a second magnesium for optimal activity(*65*−*68*).

While we propose that the results from the mutagenesis studies arise from altered K_m and K_i for cosubstrates, it is possible that M2 and PO_4 are involved in altering the rate-limiting step for enzyme turnover. High concentrations of Mg^{2+} have been shown to decrease the rate-limiting step of ADP release for other phosphotransferases (*66*). Our results provide evidence that phosphate binds near the active site affecting the binding of the substrate. This has set the stage for further studies to determine the rate-limiting step of AK and if this is affected in the presence of phosphate.

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