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Kinetic mechanism of *Toxoplasma gondii* adenosine kinase and the highly efficient utilization of adenosine

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Abstract

Toxoplasma gondii has an extraordinary ability to utilize adenosine (Ado) as the primary source of all necessary purines in this parasite which lacks *de novo* purine biosynthesis. The activity of *T. gondii* adenosine kinase (TgAK, EC 2.7.1.20) is responsible for this efficient salvage of Ado in *T. gondii*. To fully understand this remarkable efficiency of TgAK in the utilization of Ado, complete kinetic parameters of this enzyme are necessary. Initial velocity and product inhibition studies of TgAK demonstrated that the basic mechanism of this enzyme is a hybrid random bi-uni ping-pong uni-bi. Initial velocity studies showed an intersecting pattern, consistent with substrate-enzyme-co-substrate complex formation and a binding pattern indicating that binding of the substrate interferes with the binding of the co-substrate and *vice versa*. Estimated kinetic parameters were $K_{\text{Ado}} = 0.002 \pm 0.0002$ mM, $K_{\text{ATP}} = 0.05 \pm 0.008$ mM, and $V_{\text{max}} = 920 \pm 35$ $\mu\text{mol/min/mg}$ protein. Ado exhibited substrate inhibition suggesting the presence of more than one binding site for Ado on the enzyme. ATP relieved substrate inhibition by Ado. Thus, Ado also binds to the ATP binding site. AMP was competitive with ATP, inferring that AMP binds to the same site as ATP. AMP, ADP and ATP were non-competitive with Ado, therefore, none of these nucleotides binds to the Ado binding site. Combining ATP with ADP was additive. Therefore, the binding of either ATP or ADP does not interfere with the binding of the other. It is concluded that for every ATP consumed, TgAK generates three new AMPs. These findings along with the fact that a wide range of nucleoside 5'-mono, di, and triphosphates could substitute for ATP as phosphate donors in this reaction may explain the efficient and central role played by TgAK in the utilization of Ado as the major source from which all other purines can be synthesized in *T. gondii*.

Keywords

Toxoplasma gondii; Adenosine kinase; Enzyme kinetics; Purine metabolism; Parasite

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1. Introduction

Toxoplasma gondii adenosine kinase (TgAK, EC 2.7.1.20) catalyzes the phosphorylation of adenosine (Ado), to its nucleoside 5'-monophosphate, AMP, in the presence of ATP as follows:

The enzyme plays an important role in the purine metabolism of this parasite. Like other parasitic protozoa, *T. gondii* is incapable of *de novo* purine biosynthesis and depends totally on preformed purines salvaged from the host cells for its purine requirements and survival (Krug et al., 1989, Perrotto et al., 1971 and Schwartzman and Pfefferkorn, 1982). Ado is the best precursor for the synthesis of purine nucleotides in *T. gondii* and the salvage of Ado is extraordinarily efficient. Incorporation of Ado into purine nucleotides in *T. gondii* exceeds that of any other purine precursor by at least 10-fold (Krug et al., 1989 and Pfefferkorn and Pfefferkorn, 1978). Ado is converted to AMP, a branch point from which all purine nucleotides are synthesized. Metabolic, biochemical, molecular, and fitness studies demonstrated that TgAK is the route of this efficient metabolism of Ado to AMP (Chaudhary et al., 2004, Krug et al., 1989 and Pfefferkorn and Pfefferkorn, 1978). By contrast, most mammalian cells preferentially deaminate Ado to inosine; whereas, the deaminating enzyme, Ado deaminase (EC 3.5.4.4), is missing from or has very little activity in *T. gondii* (Krug et al., 1989). Moreover, comparative enzymatic, genetic, and metabolic studies showed that TgAK is the only purine nucleoside kinase in *T. gondii* (Chaudhary et al., 2004, el Kouni et al., 1999, Iltzsch et al., 1995 Krug et al., 1989, and Pfefferkorn and Pfefferkorn, 1978). TgAK does not phosphorylate any of the natural purine nucleosides other than Ado (el Kouni et al., 1999, Iltzsch et al., 1995 and Krug et al., 1989). However, TgAK, unlike human Ado kinase, can phosphorylate various 6-substituted-9-β-D-ribofuranosylpurine analogues to their respective nucleoside 5'-monophosphates (Al Safarjalani et al., 2003, 2008 and 2010, Darling et al., 1999, el Kouni, 2003 and 2007, el Kouni et al., 1999, Iltzsch et al., 1995, Kim et al, 2008 and 2010, Rais et al., 2005 and Yadav et al., 2004). When certain 6-substituted-9-β-D-ribofuranosylpurine analogues are used as subversive substrates they are selectively phosphorylated by TgAK and become toxic only against the parasites but not their host (Al Safarjalani et al., 2008 and 2010, el Kouni, 2003 and 2007, el Kouni et al., 1999, Rais et al., 2005 and Yadav et al., 2004). It follows that TgAK represents an excellent target for the development of chemotherapeutic regimens for the treatment of toxoplasmosis with 6-substituted subversive substrates (el Kouni, 2003 and 2007). Yet, in order to fully understand the remarkable efficiency of TgAK in the synthesis nucleotides of several 6-substituted-9-β-D-ribofuranosylpurine analogues as well as Ado, complete kinetic parameters of this enzyme would be necessary. Detailed kinetic studies are essential to fully understand the basic reaction mechanism of enzymes (e.g. ping-pong, sequential, random, ordered, etc.) and to illustrate the order of binding of the substrates and release of the products. The order of addition of substrates and the mechanism of action of the enzyme will shed some light on the topology of the active center. Detailed kinetic studies can also demonstrate whether either of the substrates displays substrate inhibition and/or non-linear product inhibition which would indicate

whether there is more than one binding site on the enzyme for the binding of substrate(s). Such studies are also required to determine whether there is a “cooperative effect” between the substrates or not, which would aid in illustrating whether the enzyme has multiple interacting allosteric and/or catalytic sites. Furthermore, comparison with the kinetics of human Ado kinase would reveal a great deal about the structure and function of TgAK and will also aid in understanding the differences in substrate specificities between the two enzymes. Such information cannot be visualized by x-ray crystallography of the purified enzyme but could be critical in explaining the x-ray crystallographic results and the future design of subversive substrates. Therefore, in order to fully understand the role of TgAK in the metabolism Ado and 6-substituted purines in particular, as well as for future drug design, a complete kinetic study of this enzyme is performed.

2. Materials and methods

2.1. Chemicals

[8-¹⁴C]Ado (55 Ci/mol) was purchased from Moravek Biochemicals, polyethyleneimine cellulose 300 PEI/UV₂₅₄ polygram TLC plates, from Brinkmann and bovine γ -globulin and dye reagent for protein assays were from Bio-Rad Lab. All other chemicals and compounds were obtained from Sigma Chemical Co. or Fisher Scientific. The purity of Ado, AMP, ADP or ATP used in these experiments and the lack of any contaminants was verified by HPLC analyses as we previously described (el Kouni and Cha, 1987 and el Kouni et al., 1983).

2.2. Source of TgAK

The coding sequence of cloned *TgAK* gene (Sullivan et al., 1999) was expressed and purified to homogeneity as we have previously described (Yadav et al., 2004). This purified TgAK preparation did not contain activities that could interfere with the substrates or products of the Ado phosphorylation reaction.

2.4. Enzyme assay

TgAK activity was determined in triplicates by following the formation of radiolabeled nucleotides from [8-¹⁴C]Ado (55 Ci/mol) (el Kouni et al., 1999). The standard assay mixture contained 50 mM Tris-Cl, pH 7.4, 2.5 mM of ATP, 5 mM MgCl₂, 50 μ L enzyme extract in a final volume of 100 μ L. Incubations (5–20 min.) were carried out at 37 C and terminated by boiling in a water bath for 2 min, followed by freezing for at least 20 min. Precipitated proteins were removed by centrifugation and 10 μ L of the supernatant was spotted on 300 PEI/UV₂₅₄ polygram plates. The plates were first developed in H₂O then in 1 M lithium chloride. In this system, the R_f values were: Ado, 0.80; AMP, 0.51; ADP, 0.28; ATP, 0.11. A computerized Berthold LB-284 Automatic TLC-Linear Analyzers (Berthold Inc., Gaithersburg, MD) was used to detect the radioactive spots and calculate the amounts of radioactivity in the substrate and product(s) on a percentage basis. Thin layer chromatographic separation of the assay supernatant showed only two radioactive spots corresponding to the substrate (Ado) and product (AMP). Specific activity is calculated as the μ moles/mL product formed, as computed from the net %conversion of Ado to AMP,

divided by the minutes of incubation and the mg of enzyme protein/mL. Protein concentrations were determined by the method of Bradford (Bradford, 1976)

2.5. Kinetic studies

Kinetic determinations were run under the standard assay conditions, with adjustments to varying ligand concentrations as to insure strict linearity with incubation time and enzyme concentration. Kinetic parameters were estimated by computer programs employing the general methods of Wilkinson (Wilkinson, 1961) and Cleland (Cleland, 1967) as previously described [el Kouni et al., 1999 and Iltzsch et al., 1985). All kinetic parameter values represent means $\pm$ S.E.

3. Results and discussion

3.1. Substrate specificity

TgAK phosphorylates Ado to the exclusion of any other natural purine nucleoside (el Kouni et al., 1999, Iltzsch et al., 1995 and Krug et al., 1989). However, the enzyme can also phosphorylate several 6- and 7-substituted Ado and inosine analogues to their respective 5'-monophosphate derivatives (Al Safarjalani et al., 2003, 2008 and 2010, Darling et al., 1999, el Kouni, 2003 and 2007, el Kouni et al., 1999, Iltzsch et al., 1995, Kim et al, 2008 and 2010, Rais et al., 2005 and Yadav et al., 2004). Thus, TgAK is not strictly specific for Ado. To determine the specificity of the nucleoside 5'-triphosphate co-substrate, TgAK activity was determined with various nucleoside 5'-triphosphate donors. Table 1 shows that TgAK can use various nucleoside 5'-triphosphates as phosphate donors, albeit to different degrees. The activity was highest with ATP or GTP and lowest with TTP. Therefore, TgAK appears to display broad specificities towards both the substrate, Ado, and the co-substrate, ATP. Nonetheless, since ATP is the conventional phosphate donor, and TgAK activity with ATP or GTP, are more or less equivalent, ATP was used as the co-substrate in all the present studies on this enzyme. TgAK also requires Mg^{++} for its activity. No activity was observed in the absence of Mg^{++} . However, Mg^{++} may be inhibitory at concentrations above the optimum for a given ATP concentration (data not shown).

3.2. Effect of substrate concentration

The apparent K_{Ado} was 0.006 ± 0.0008 mM as estimated from varied Ado concentrations and a fixed ATP concentration (2.5 mM). The activity increased with the increase of Ado concentrations up to 0.02 mM ($3 \times$ the apparent K_{Ado}), then gradually decreased (Fig. 1), indicating substrate inhibition by Ado. This suggests the presence of more than one binding site for Ado on the enzyme. This finding was sustained at various other non-saturating fixed concentrations of ATP (0.03–0.09 mM) (Fig. 1). These results indicate that non-saturating ATP concentrations led to a pronounced substrate inhibition by Ado, at concentrations that are not inhibitory in the presence of saturating ATP concentrations. This finding suggests that the second binding site for Ado is, likely, the ATP binding site itself, since, increasing the ATP concentration seems to override substrate inhibition by Ado, as denoted by the shift of the trough toward the right in Fig 1. Indeed, crystallographic studies indicated that Ado binds to both the Ado and ATP binding sites (Zhang et al., 2006).

The apparent K_{ATP} was 0.04 ± 0.004 mM as estimated from varied ATP (0.02–0.2 mM) concentrations and a fixed Ado concentration (0.015 mM). No substrate inhibition was observed with increasing ATP concentrations up to 0.2 mM ($5 \times$ the apparent K_{ATP}). The apparent K_{ATP} is much larger than K_{Ado} , indicating that ATP has lower affinity for the enzyme than Ado.

3.3. Kinetic Studies

3.3.1. Initial Velocity Studies—The plots of $1/v$ vs. $1/[Ado]$ and $1/v$ vs. $1/[ATP]$ showed an intersecting pattern, with a common intersection at coordinates $1/Ado = -1216$, $1/ATP = -56$ and $1/v = -2000$, indicating a sequential mechanism, whereby all substrates must bind to the enzyme before any product is released. This concurs with the results of our crystallographic studies of TgAK (Zhang et al., 2006). The results of initial velocity studies with varied Ado (0.0005–0.01 mM) and various changing fixed concentrations of ATP (0.375–0.9 mM) are presented in Fig. 2. With variable $[Ado]$ and increasing fixed $[ATP]$ (Fig. 2) as well as with variable $[ATP]$ and increasing fixed $[Ado]$ (plots not shown), there was an increase in apparent K_{Ado} and K_{ATP} , respectively. Consequently, the binding of one substrate seems to decrease the binding of the other, *i.e.*, the binding of ATP decreases the affinity of the enzyme for Ado and *vice versa*. Replot of slopes vs. $1/[ATP]$ was non-linear (Fig. 2) indicating a possibility of a hybrid steady-state (random or ordered) and a ping-pong mechanism. Assuming linearity, nonetheless, the replots yielded $K_{Ado} = 0.002 \pm 0.0002$ mM, $K_{ATP} = 0.05 \pm 0.008$ mM, and $V_{max} = 920 \pm 35$ μ mol/min/mg protein. These kinetic parameters are necessary for analysis and determination of the reaction mechanism of TgAK. It is to be noted that the deviation from linearity of the replots, led to approximation of these parameters. For example, the intersection with the ordinate from which the V_{max} value is determined is a function of the incline of the tangent to the intercepts curve in the replot, which incline depends on the points making the tangent. We, herein, adhered to the incline dictated by the least square fit, as previously described (Chang et al., 1983, el Kouni et al, 1988 and Iltzsch et al., 1985).

3.3.2. Product Inhibition Studies with varied concentration of Ado and fixed concentration of ATP

3.3.2.1. AMP as Product inhibitor with a saturating concentration of ATP: Fig. 3 shows that the plot of $1/v$ vs. $1/[Ado]$ (Ado varying from 0.003 to 0.018 mM), at a single fixed saturating concentration of ATP (0.5 mM) and various fixed concentrations of AMP (0–0.4 mM), is an intersecting pattern, with a common intersecting point at coordinates $1/Ado = -88 \pm 15$ and $1/v = 0.00012 \pm 0.00001$. The inhibition constant, $K_{ii} = 0.53 \pm 0.05$ mM, was estimated from the replot of $1/v$ -intercepts vs. $[AMP]$. Although Ado is phosphorylated to AMP at the Ado binding site, added AMP inhibited Ado non-competitively. This indicates that AMP does not bind the Ado binding site. This is corroborated by the initial velocity results showing that Ado binding decreased the affinity of the enzyme for ATP and *vice versa*, suggesting that ligand binding may induce enzyme conformational changes, which could interfere with the binding of other ligands. This is in agreement with our crystallographic studies of TgAK (Zhang et al., 2006) which demonstrated that Ado binding and ATP binding are two independent processes associated with separate conformational changes.

3.3.2.2. AMP as Product Inhibitor with a non-saturating concentration of ATP: Fig. 4 shows that the plot of $1/v$ vs. $1/[Ado]$ (Ado varying from 0.003 to 0.018 mM), at a single fixed non-saturating concentration of ATP (0.05 mM) and various fixed concentrations of AMP (0–0.8 mM), is an intersecting pattern, with two common intersecting points. One point at a low range of Ado concentrations (0.003–0.009 mM) intersecting at coordinates $1/Ado = -664$ and $1/v = 0.00003$. The second point at a higher range of Ado concentrations (0.012–0.018 mM) intersecting at coordinates $1/Ado = 26.5$ and $1/v = 0.0001$. Values of inhibition constants, estimated from replots of $1/v$ -intercepts vs. $[AMP]$ were $K_{ii} = 0.22 \pm 0.06$ mM, at low (0.003–0.009 mM), and $K_{ii} = 0.32 \pm 0.2$ mM, at high (0.012–0.018 mM) Ado concentrations (replots not shown). The two intersecting patterns are indicative of the occurrence of two distinct reactions. Since, this only happened under non-saturating ATP concentration (0.05 mM), it is possible that the second intersecting pattern reflects the formation of a productive Ado-enzyme-AMP complex. In any case, the product, AMP, seems to inhibit Ado non-competitively. Hence, added AMP does not bind to the Ado binding site.

3.3.2.3. ADP as product inhibitor with saturating and non-saturating concentrations of ATP: Fig. 5 shows that the plot of $1/v$ vs. $1/[Ado]$ (Ado varying from 0.003 to 0.018 mM), at a single fixed saturating concentration of ATP (0.5 mM) and various fixed concentrations of ADP (0–0.8 mM), is an intersecting pattern, with a common intersecting point at coordinates $1/Ado = -102 \pm 17$ and $1/v = 0.000098 \pm 0.000016$. K_{ii} value estimated from the replot of $1/v$ -intercepts vs. $[ADP]$ was 1.00 ± 0.09 mM. The plot of $1/v$ vs. $1/[Ado]$, under identical assay conditions but at non-saturating ATP (0.05 mM), was also typically non-competitive (plot not shown). Consequently, neither AMP nor ADP and by extrapolation nor ATP binds to the Ado binding site on TgAK.

3.4. Product Inhibition Studies with varied concentration of ATP and fixed concentration of Ado

3.4.1. AMP as Product inhibitor with saturating concentration of Ado—Fig. 6 shows that the plot of $1/v$ vs. $1/[ATP]$ (ATP varying from 0–0.3 mM), at a single fixed saturating concentration of Ado (0.018 mM) and various fixed concentrations of AMP (0–0.4 mM), is an intersecting pattern, with a common intersecting point at coordinates $1/ATP = 0.00$ and $1/v = 0.003 \pm 0.0002$, indicating that AMP competitively inhibited the reaction. K_{is} value, estimated from the replot of slopes vs. $[AMP]$, was 0.16 ± 0.04 mM. Thus, AMP seems to bind into the ATP binding site. This corroborates our proposition of formation of a productive Ado-enzyme-AMP complex, as stated above for the results depicted in Fig. 4.

3.4.2. AMP as Product inhibitor with non-saturating concentration of Ado—At non-saturating concentration of Ado (0.005 mM), the effect of AMP at high concentration (1.9 mM) acted as if it were additive to that of ATP as a phosphate donor (data not shown). Thus, it appears that the non-saturating concentrations of Ado improves the chances of AMP to bind to the ATP binding site and subsequently acts as a phosphate donor when Ado binds to the enzyme.

3.4.3. ADP as Product Inhibitor at saturating concentration of Ado—Fig. 7

shows that not only does ADP not inhibit the phosphorylation of Ado by ATP but, in fact, it increases the velocity of phosphorylation in a dose dependent manner. There was almost perfect correlation between doubling the ADP concentration and halving of the distance between the activity lines. It is as if phosphorylation by ADP superseded that by ATP in the reaction. The range of increase between activities with and without addition of ADP to ATP is compatible with an additive effect, rather than inhibition. These results, somewhat bear resemblance to the data obtained with AMP as product inhibitor with a non-saturating concentrations of Ado, whereby the effect of higher concentrations of AMP (1.9 mM) were additive to that of ATP as a phosphate donor.

Since the range of AMP or ADP concentrations used are not compatible with AMP or ADP being activators, we were led to explore the capacity of AMP, ADP, ATP, dADP, GDP or UDP as phosphate donors (Table 2). Table 2 shows that in the absence of a phosphate donor, there was no phosphorylation of Ado. In contrast, ATP, all of the nucleoside 5'-diphosphates used, and AMP acted as phosphate donors for the phosphorylation of Ado, albeit to different degrees. $UDP < GDP < dADP < ADP$, and to a lesser extent AMP, replaced ATP as phosphate donors. These findings demonstrate that TgAK can phosphorylate Ado in the presence of $AMP \ll ADP < ATP$.

Therefore, it appears that in our assay, radiolabeled ^{14}C -Ado with ATP, would yield two ^{14}C -AMP, as follows:

On the other hand, radiolabeled ^{14}C -Ado with unlabeled ADP ($^{14}C-Ado + ADP \rightarrow ^{14}C-AMP + AMP$) or AMP ($^{14}C-Ado + AMP \rightarrow ^{14}C-AMP + Ado$) would yield only one ^{14}C -AMP.

Hence, we propose that TgAK catalyzes the conversion of Ado to AMP, independently or stepwise as follows:

Although the present results have demonstrated that reaction (3) is plausible *in vitro*, this reaction may be negligible *in vivo*, since the concentration of available AMP ($K_{ii} = 0.053$ mM) resulting from the dephosphorylation of ATP and/or ADP would not support the phosphorylation of Ado. In addition, the conversion of Ado to AMP with AMP as a phosphate donor is futile since the products, Ado and AMP, are identical to the substrates, Ado and AMP, the reaction started with. For a biological system that has evolved into such

efficiency, reaction (3) may not occur, or occurs very little in nature. Therefore, it can be safely concluded that TgAK generates, from every ATP consumed, three new AMPs produced by reactions (1) and (2). In an organism like toxoplasma, devoid of *de novo* purine biosynthesis, and in which all purines required for survival are mainly synthesized from AMP, this process of amplification of AMP formation is seminal and could be the reason behind the extraordinary efficiency of TgAK and its central role in the purine metabolism of *T. gondii*.

4. Conclusions

The present initial velocity studies indicate that the double reciprocal plot of TgAK yields a non-competitive pattern, while the product inhibition studies with fixed changing AMP yields a competitive pattern with $1/v$ vs. $[1/ATP]$ at a fixed Ado concentration and a non-competitive pattern with $1/v$ vs. $1/[Ado]$ at a fixed ATP concentration as summarized in Table 3. These results are compatible with a random system (Segel, 1993).

In light of these results, we propose that the TgAK reaction mechanism is a hybrid random bi-uni ping-pong uni-bi, as depicted below:

Our present results clearly indicate that the mechanism of action of TgAK is random not ordered. It is more plausible that whenever ATP and Ado enter their respective sites, ATP donates the γ -phosphate to Ado. The formed AMP is readily released from the Ado site as its K_{ii} is 0.5 mM. However, before the ADP, produced from the dephosphorylation of ATP, is released, a second Ado enters the vacant Ado site and is instantaneously phosphorylated by the enzyme-bound-ADP, as inferred from the almost perfect correlation between doubling the ADP concentration and halving of the distance between the activity lines, from which it might be construed that ADP supplants ATP in the reaction (Fig. 7). Thus, the order of occupancy of the Ado site is not that Ado always enters before the phosphate donor. It may enter first when ATP is the phosphate donor, but it is certainly second in the order of occupancy when the ADP, produced from ATP dephosphorylation, is the phosphorylating agent. This is precisely the reason that the TgAK reaction is not an ordered, but rather a random mechanism, as clearly indicated by the present results (Table 3). This is further supported by direct evidence as crystallographic studies (Zhang et al., 2006) showed that Ado binding and ATP binding are two independent processes associated with separate conformational changes. Furthermore, in all the available TgAK structures studied (Zhang et al., 2007), no structure was seen with an occupied nucleoside-binding site and an empty ATP-binding. only when Ado is present, it occupied both the ATP and nucleoside-binding sites.

Moreover, our results indicate that ADP is by far a better phosphate donor than AMP (Table 2). Yet, the bindings of AMP and ATP are mutually exclusive, whereas the bindings of ADP and ATP are additive (Fig. 7). The multiplicity of the phosphate donors in the TgAK

reaction (Tables 1 & 2), suggest that all the phosphate donors bind to a single site. In consequence, it is plausible that additivity results from a reaction with ADP as depicted above. Notwithstanding ATP binding before Ado or *vice versa*, it is possible that the release of ADP from the ATP site is rate limiting, i.e., without this release no further catalysis by ATP is possible. Indeed, the rate of an enzymic reaction is governed by binding of substrate and/or release of product. Hence, the binding of Ado may hinder the binding of ATP and *vice versa* (Fig. 2). But the efficiency of TgAK attests that this effect is likely a result of conformational changes associated with the ligand binding process, which in no way hinders catalysis. Our results also indicate that AMP is readily releasable from the Ado site as its K_{ii} is 0.5 mM; therefore, the release of ADP must be the key determinant of further catalysis. The fact that we could not obtain any inhibitory pattern with ADP as product inhibitor of the TgAK reaction with varied ATP, indicates that the bindings of ATP and ADP are not mutually exclusive (Fig. 7) as both ATP and ADP bind to a single site (Zhang et al., 2006), is a sufficient proof for this proposition.

In conclusion, the mechanism of TgAK is a hybrid random bi-uni ping-pong uni-bi as depicted in the above scheme. Thus, the overall reaction of TgAK could be portrayed as the sum of two partial reactions in which enzyme bound ADP acts as an intermediate phosphate donor as follows:

A steady-state treatment of the proposed mechanism is too complex to make possible derivation of a rate equation. An equation based on rapid equilibrium, using the method of Cha (Cha, 1968) would have been quite manageable. A requisite of rapid equilibrium, however, is that the binding of one ligand has no effect on the binding of another. Our initial velocity studies clearly indicate that the binding of Ado decreases the binding of ATP and *vice versa*, which precludes resorting to rapid equilibrium. Derivation of rate equation for this reaction must hence await the development of computer programs that could solve such steady state equation.

In retrospect, the decrease in binding of a substrate upon binding the other, suggestive of enzyme conformational changes, may have evolved to ensure the occurrence of reaction (2), thus increasing the overall efficiency of TgAK.

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Abbreviations

Ado	adenosine
K_{ii}	the inhibition constant computed from the replot of intercepts

K_{is}	the inhibition constant computed from the replot of slopes
TgAK	<i>Toxoplasma gondii</i> adenosine kinase [EC 2.7.1.20]
TLC	thin layer chromatography

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Fig. 1.
Substrate inhibition by Ado at varied ATP concentrations (0.03, 0.06, 0.09 and 2.5 mM).

Fig. 2.

Initial Velocity Studies. Plot of $1/v$ vs. $1/[\text{Ado}]$ at various fixed concentrations of ATP and replots of $1/v$ -intercepts and slopes vs. $1/[\text{ATP}]$. The common intersection point was estimated at coordinates $1/\text{Ado} = -1216$, $1/\text{ATP} = -56$ and $1/v = -2000$, $K_{\text{Ado}} = 0.002 \pm 0.0002$ mM, $K_{\text{ATP}} = 0.05 \pm 0.008$ mM, and $V_{\text{max}} = 920 \pm 35$ $\mu\text{mol/min/mg protein}$ were estimated from the replots.

Fig. 3.

Product Inhibition by AMP at varied concentrations of Ado (0.003–0.018 mM) and a fixed saturating concentration of ATP (0.5 mM). Plot of $1/v$ vs. $1/[Ado]$ at various fixed concentrations of AMP (0–0.4 mM) and replots of $1/v$ -intercepts and slopes vs. $[AMP]$. $K_{ii} = 0.53 \pm 0.05$ mM as estimated from the replot of $1/v$ -intercepts.

Fig. 4.

Product Inhibition by AMP at varied concentrations of Ado (0.003–0.018 mM) and fixed non-saturating concentrations of ATP (0.05 mM). Plot of $1/v$ vs. $1/[Ado]$ at various fixed concentrations of AMP (0–0.8 mM). At 0.003–0.009 mM Ado, $K_{ii} = 0.22 \pm 0.06$ mM as estimated from replot of $1/v$ -intercepts vs. [AMP]. And at 0.012–0.018 mM Ado, $K_{ii} = 0.32 \pm 0.20$ mM as estimated from replot of the $1/v$ - intercepts vs [AMP]. Replots of $1/v$ -intercepts and slopes are not shown.

Fig. 5.

Product Inhibition by ADP at varied concentrations of Ado (0.003–0.018 mM) and a fixed saturating concentration of ATP (0.5 mM). Plot of $1/v$ vs. $1/[Ado]$ at various fixed concentrations of ADP (0–0.8 mM) and replots of $1/v$ -intercepts and slopes vs. $[ADP]$. $K_{ii} = 1.0 \pm 0.09$ mM was estimated from the replot of the $1/v$ -intercepts vs. $[ADP]$.

Fig. 6.

Product Inhibition by AMP at varied concentrations of ATP (0.01–0.3 mM) and fixed concentration of Ado (0.018 mM). Plot of $1/v$ vs. $1/[ATP]$ at various fixed concentrations of AMP (0–0.4 mM) and replots of $1/v$ -intercepts and slopes vs. [AMP]. $K_{is} = 0.16 \pm 0.04$ mM as estimated from the replot of the slope vs. [AMP].

Fig. 7. Effects of adding ADP to ATP on the activity of *T. gondii* adenosine kinase. Plot of V_{max}/v vs. $K_{ATP}/[ATP]$ at varied concentration of ATP (0.01–0.4 mM), fixed Ado concentration of 0.018 mM and various fixed concentrations of ADP (0–0.4 mM).

Table 1

Percent conversion of Ado (15 μ M) to AMP, after 15 min. incubation with *T. gondii* adenosine kinase in the presence of 0.1 mM of various nucleoside 5'-triphosphate donors

Phosphate donor	% conversion
GTP	38.4 $\pm$ 0.2
ATP	36.2 $\pm$ 1.4
dGTP	30.8 $\pm$ 1.8
dATP	34.5 $\pm$ 1.6
CTP	26.6 $\pm$ 4.7
UTP	18.7 $\pm$ 2.8
dCTP	18.9 $\pm$ 5.9
TTP	15.1 $\pm$ 4.4

Values are means $\pm$ SD from at least two experiments of three replica each.

Table 2

Percent conversion of Ado (0.018 mM) to AMP by *T. gondii* adenosine kinase, after 10 min. incubation in the presence of (0.4 mM) of various nucleoside 5'-mono, di, and triphosphate donors.

Phosphate donors	% Conversion
None	0
ATP	96.2 ± 0.3
ADP	47.7 ± 3.6
AMP	1.9 ± 0.5
dADP	28.5 ± 2.0
GDP	21.1 ± 2.4
UDP	5.9 ± 1.0

Values are mean ± SD from at least two experiments of three replica each.

Table 3Summary of Initial velocity and Product inhibition of *T. gondii* adenosine kinase

Control Reciprocal Plots		Reciprocal Plots with AMP as an Inhibitor	
$1/v$ vs. $1/[Ado]$ at various fixed $[ATP]$	$1/v$ vs. $1/[ATP]$ at various fixed $[Ado]$	$1/v$ vs. $1/[Ado]$ at fixed $[ATP]$	$1/v$ vs. $1/[ATP]$ at fixed $[Ado]$
non-competitive	non-competitive	mixed-type	competitive