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Differential Positioning of Functional Cysteine Residue(s) Determines Catalytic Characteristics of Adenosine Kinase from Divergent Sources

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Dissimilar catalytic characteristics of adenosine kinase (E.C. 2.7.1.20), isolated from the parasitic protozoan *Leishmania donovani* and its natural host hamster, have been investigated with the help of protein modification experiments. Incubation of adenosine kinase from both the sources with several thiol-group blockers led to rapid loss of enzyme activity with pseudo first-order kinetics. However, the degree of sensitivity of the enzymes to various cysteine-directed reagents differed significantly. Adenosine and its analogs afforded differential protection to both the enzymes against −SH blocker mediated inactivation, indicating localization of cysteine residues at the Ado binding site. Based on differential protection experiments we show that, in contrast to leishmanial adenosine kinase, the enzyme from hamster contained two Ado binding sites. Of these, one is the high affinity catalytic site while the other binds Ado with lower affinity and is catalytically inactive. Evidence is also presented to indicate that the low affinity Ado binding site of hamster enzyme, which contains at least one −SH group, is the site responsible for both protection against −SH blocker mediated inactivation and substrate inhibition caused by Ado. In contrast, in leishmanial enzyme, the catalytic and protective sites are overlapping. Thus, the functional −SH group(s) in two enzymes appear to take part in the respective reactions by differential mechanisms. Possible biological implications of these findings are discussed.

Key Words: SH-blockers, Substrate protection, Substrate inhibition, Liver, *Leishmania donovani*

Introduction

Selective chemotherapy of infectious diseases can be attained by compounds that inactivate novel enzymes necessary for replication of the invading organisms leaving host processes unaffected. Hence, designing of metabolic inhibitors requires an indepth knowledge of the enzymes involved in the process.

Adenosine kinase, one of the purine salvage enzymes, which directly phosphorylates adenosine to AMP, has been implicated in the phosphorylation of many pharmacologically active purine nucleoside

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analogs (Kornberg & Pricer 1951, Murray et al. 1970, Iovannisci et al. 1984). Despite the fact that this enzyme is abundant and almost ubiquitous in nature, its physiological function is yet to be well understood. Earlier studies from this laboratory have indicated that adenosine kinase from *L. donovani*, unlike eukaryotic adenosine kinase, displays distinctly unique kinetic and immunological properties (Datta et al. 1987, Bhaumik & Datta 1989). The substrate recognition characteristics of leishmanial enzyme also differ from that observed with other mammalian adenosine kinases (Bhaumik & Datta 1989, Bhaumik & Datta 1988).

High concentrations of adenosine has been shown to inhibit adenosine kinase of most eukaryotic sources (Datta et al. 1987, Bhaumik & Datta 1988, Bhaumik & Datta 1989). However, adenosine kinase from *L. donovani* is insensitive to excess adenosine. In search of answer to question on why leishmanial enzyme, as opposed to other eukaryotic adenosine kinases, is not sensitive to excess Ado, we have undertaken a detailed comparative investigation on the role of different amino acid residues which are critically connected with the catalytic activity. Recently, we have demonstrated that the parasite enzyme harbors two essential cysteine residues at or near the active site (Bhaumik & Datta 1992). Adenosine kinase from hamster, a natural host for *L. donovani*, is also susceptible to −SH blockers. This communication reports that, in contrast to the parasite enzyme, the reactive thiol group(s) of hamster enzyme is located at a discrete site distal from the catalytic center. Binding of Ado at this site leads to both substrate inhibition and protection of the enzyme. A plausible hypothesis is being proposed with regard to the differential functions of reactive thiol group(s) of the enzyme from two sources.

Materials and Methods

Reagents : N-ethylmaleimide (NEM), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Methylmethane · thiosulfonate (MMTS), *p*-hydroxymercuribenzoate (pHMB), and 6-methylmercaptopurine riboside (6-MMPR) were the products of Sigma Chemical Company. Sources of other chemicals and strains were as described in earlier publications (Datta et al. 1987).

Enzyme assays : Both radiochemical and spectrophotometric assays were carried out following the procedure described before (Datta et al. 1987, Bhaumik & Datta 1989). Unless otherwise stated, for assay of leishmanial enzyme, the concentrations of Ado and Mg^{++} were 50 μM and 1.5 mM while hamster enzyme was assayed using 15 μM and 0.5 mM of Ado and Mg^{++} respectively. The time-dependent linearity of all plots was checked graphically for all substrate concentrations. For determination of initial velocities, kinetic data were fitted into Michaelis-Menton equations, i.e. rectangular hyperbola and double reciprocal plots were done according to Cleland (1970).

Inactivation with −SH blockers : Incubations for determining the rate of enzyme inactivation were performed at 30°C in a volume of 50μl. Each incubation contained 50 mM Tris *p*H 7.5, 1 mg/ml BSA, 20% glycerol, appropriate amounts of purified enzyme, and given concentrations of ligand. Where inactivation was caused by −SH blockers, the reagent was added at zero time. Enzyme activity remaining after various time intervals, was determined after proper dilution of the enzyme. When various concentrations of ligands were used, the assay was modified accordingly to maintain proper substrate concentration.

Determination of kinetic constants : "Dissociation constant (K_s)" for enzyme-substrate complex was determined kinetically (Cleland 1970). "Protection

constant (K_p)" is defined as the concentration of ligand required for 50% protection of the enzyme activity against inactivation caused by the inactivator molecule (Chuang & Bell 1972). Under conditions, where protective ligand is the natural substrate, K_p value can be equated to K_s.

Inactivation of the enzyme in presence of varying concentrations of protecting compounds followed a first-order kinetics. From a plot of log (percentage of remaining activity) against time, the rate constants of inactivation were determined over a range of protector concentrations including zero. K and K_o denote rate constants in presence and absence of protective ligand.

Purification of enzymes: Adenosine kinase from *L. donovani* and hamster liver were purified following the procedures described before (Datta et al. 1987, Bhaumik & Datta 1992).

Results

Properties of hamster enzyme : Molecular weight of adenosine kinase varies from source to source (Miller 1979, Chang et al. 1980, Yamada et al. 1982). The purified enzyme from hamster liver exhibited a molecular weight of about 44,000 which turned out to be different than the enzyme from *L. donovani* (Datta et al. 1987). The activity of hamster enzyme was stimulated by increasing concentration of KCl reaching a maximum at around 400mM. In contrast, enzyme from *L. donovani* was inhibited by KCl. Thus ionic factors probably play some role during expression of hamster enzyme activity while this did not seem to be the case for leishmanial enzyme.

Effect of sulfhydryl group-specific reagents — Reaction with NEM, pHMB and DTNB : Adenosine kinase from several sources was shown to be sensitive to −SH group specific reagents (Miller et al. 1979, Chang et al. 1980, Andres & Fox 1979). Sensitivity of leishmanial enzyme to NEM was also demonstrated in an earlier publication (Bhaumik & Datta 1989). In the present work, a comparative study has been undertaken to assess the effects of several −SH group blockers on adenosine kinase from hamster and leishmania. Results presented in figure 1(A,B,C) indicate the time course of inactivation of enzyme from both sources. Enzyme from either sources, when exposed identically to different −SH group specific reagents, exhibited distinct but differential sensitivities. The rates of inactivation of both the enzymes in presence of NEM, pHMB and DTNB followed pseudo first-order kinetics. In presence of NEM, leishmanial enzyme was found to be inactivated at a slightly faster rate than hamster enzyme. On the contrary, pHMB and DTNB inactivated hamster enzyme much faster. The differential inhibitory effects of these agents on enzymes from both sources were also manifested in the results presented in figure 1(a, b, c). In the presence of increasing concentration of NEM, the leishmanial enzyme was inhibited to a slightly greater extent than hamster enzyme. However, the extent of inhibition of the two enzymes were just opposite when the experiments were carried out with pHMB and DTNB. Differential reactivity of the two enzymes to −SH blockers thus indicated structural differences between the enzymes.

Substrate protection : In an attempt to localize the site of essential cysteine residue(s) in two enzymes, protection from inhibition by Ado was examined. The results of substrate protection studies, plotted as first-order decay curves are shown in figure 2. With NEM as the modifying reagent, Ado afforded much higher protection to leishmanial enzyme than the enzyme from hamster. In this connection, it must be noted that the K_m of Ado for leishmanial enzyme (16μM) is much higher

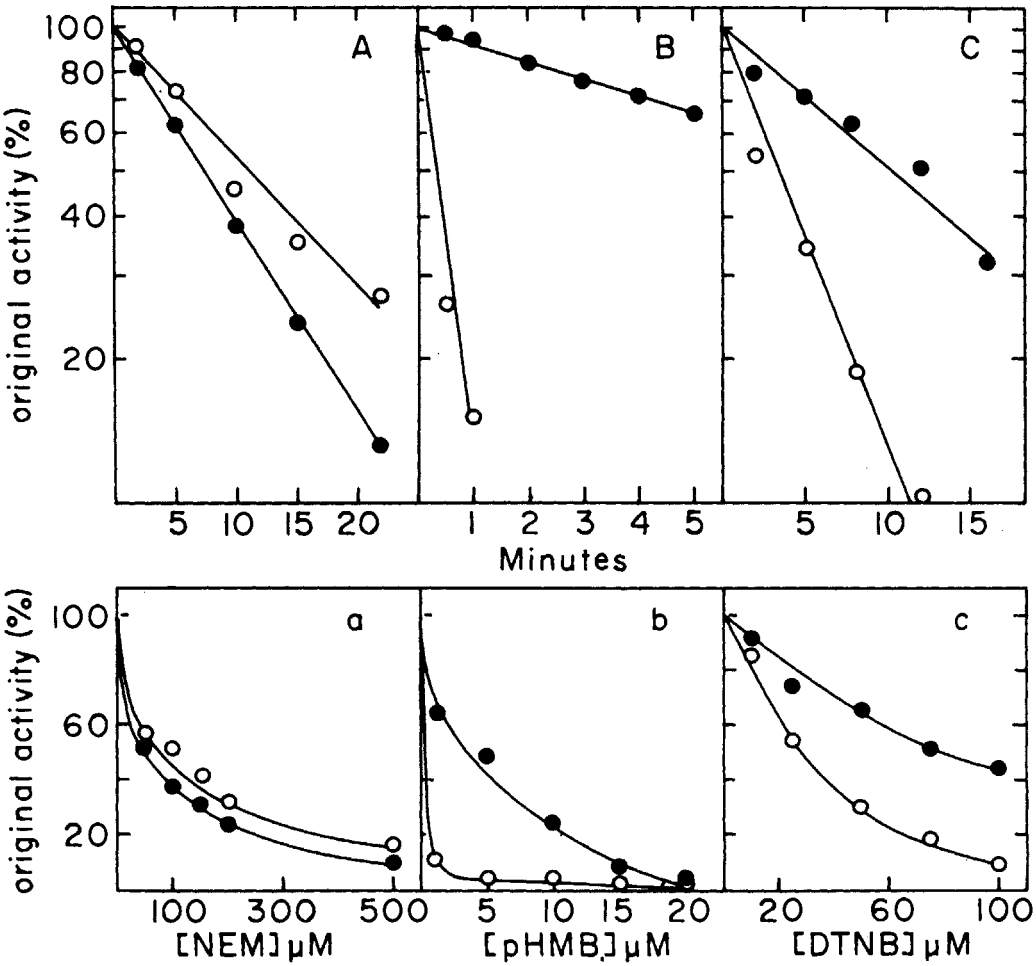

Figure 1 Effects of NEM, pHMB and DTNB on the phosphorylation of adenosine by leishmanial (●) and hamster liver (○) adenosine kinase. Extents of inactivation of adenosine kinase by (A), NEM (0.2mM), (B) pHMB (0.002mM) and (C) DTNB (0.1mM) are presented. Enzyems were preincubated with reagents at 30°C. At various time intervals, aliquots were removed, diluted and assayed for adenosine phosphorylating abilities. Results presented in lower panel show the inhibition of enzyme activity in the presence of varying concentrations of (a) NEM, (b) pHMB, (c) DTNB. Enzymes were preincubated with indicated concentrations of NEM, pHMB and DTNB for 5', 5' and 12' respectively. Aliquots were removed, diluted and assayed as usual.

than the counterpart hamster enzyme (2.8μM).

To ascertain if protection against NEM modification reflects binding of Ado to a single site, the K_p for Ado was determined by measuring inactivation rates as a function of Ado concentration as described by Chuang and Bell (1972). The plot of $1/K_o$-K against 1/Ado is linear and yields values of K_p of about 3.4 and 140μM respectively for

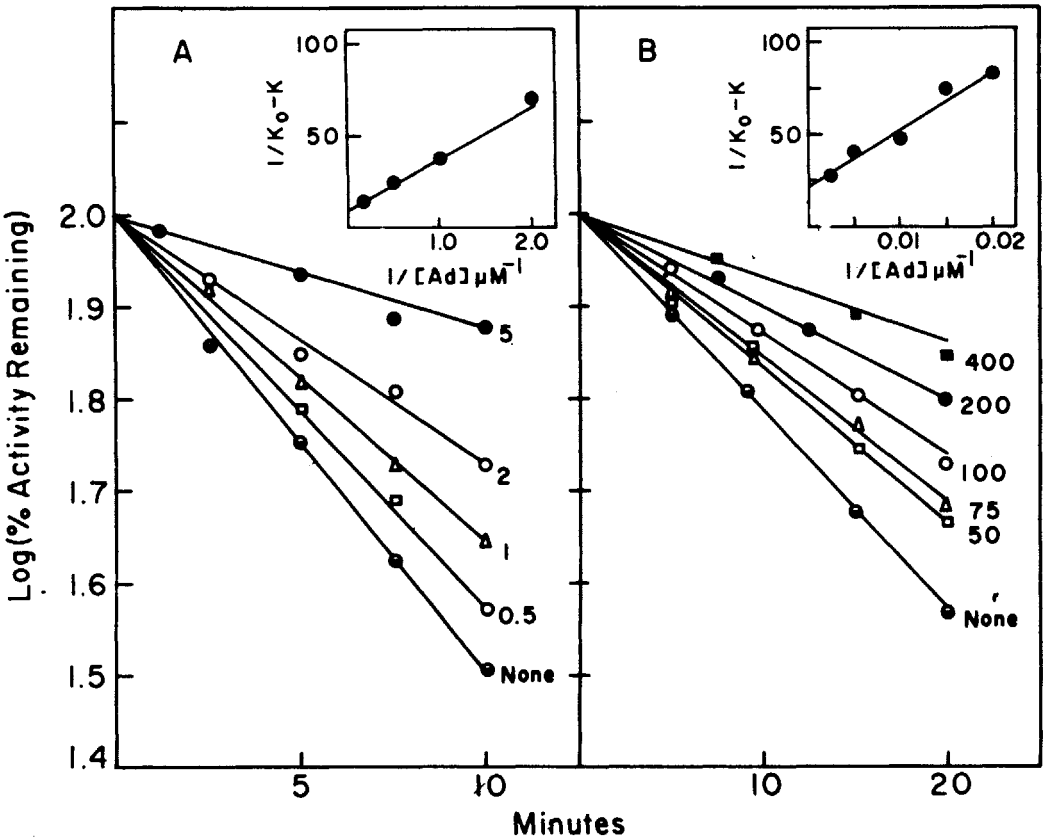

Figure 2 Protection by adenosine against NEM-mediated inactivation of adenosine kinase from leishmania (A) and hamster liver (B). Micromolar concentrations of Ado, as indicated, were used as the protecting ligand. Determination of protection constants (K_p) for adenosine from the primary data are shown in the inset.

leishmanial and hamster enzymes (figure 2, inset). To equate between K_p and K_s, the dissociation constant (K_s) for ES complex of the two enzymes were also determined. The K_s for leishmanial and hamster enzymes were 2.7 and 1.7μM respectively. The results of these experiments thus indicated a common site of binding for NEM and Ado for leishmanial enzyme, which obviously is the catalytic site. On the contrary, in hamster enzyme, NEM binds to the enzyme at a site discrete from the catalytic site. Nevertheless, the binding of NEM to both the enzymes leads to loss of enzyme activity.

Protection by adenosine analog : 6-MMPR, an excellent alternative substrate and a competitive inhibitor of adenosine phosphorylation, has been shown to interact with the placental adenosine kinase mostly at the catalytic site (Lin et al. 1988). Moreover, when used as the substrate, 6-MMPR elicits weak inhibitory effect on hamster adenosine kinase (figure 3). It was thus of interest to

check the protective effect of this ligand towards the rate of NEM-mediated inactivation of the enzymes from two sources. Results presented in table 1 indicate that prior presence of 6-MMPR during NEM treatment protected leishmaial enzyme to the extent of 71%, while the enzyme from hamster, under identical conditions, was inactivated almost identically both in the presence and absence of 6-MMPR. It must be pointed out that the concentration of 6-MMPR (100 μM) used in these protection experiments far exceeded its K_m of 2.8μM (Bhaumik & Datta 1988). Apparently, lack of binding of 6-MMPR to the protective site of hamster enzyme accounted for inactivation of the enzyme.

Discussion

Differential catalytic behaviour of adenosine kinase from various sources are well documented (Miller et al. 1979, Chang et al. 1980, Andres & Fox 1979, Bennett et al. 1978). However, the exact biochemical basis for this differential behaviour is yet to be well understood. In order to delineate the mechanism, a comparative study, involving adenosine kinase from hamster as an eukaryotic model and the enzyme from leishmanial parasite, was undertaken.

The data presented above support the following major conclusions: (i) adenosine kinase from both hamster liver and leishmanial parasite are sensitive to cysteine blockers, nevertheless the extents of reactivity of the blocker towards both the enymes differ markedly, (ii) hamster enzyme, as opposed to the parasite enzyme, contains two adenosine binding sites with differential affinities for Ado, (iii) Low affinity adenosine binding site, which is catalytically inactive, contains at least one reactive −SH group, (iv) Ado and its analogs protect the two enzymes differentially against NEM-mediated inactivation and (v) Ado, at high concentrations, reversibly inhibits hamster adenosine kinase.

Exposure of adenosine kinase from both the sources with a variety of cysteine modifying agents including NEM, pHMB and DTNB resulted in differential loss of enzyme activity. Since these agents are known to be bulky in size, the differential effects observed could be due to inaccessibility of the ragents to the site (Smith et al.1975, Kennedy et al. 1988). However, inhibition of the enzyme activity by methylmethane thiosulfonate ruled out such possibilities (Bhaumik & Datta 1992). Thus, the thiol groups of the two enzymes seem to be involved during substrate binding or catalysis.

Is the essential thiol at the active site?: Loss of enzyme activity by thiol-specific reagents may be due to modification of an active site thiol, or to modification of a nonactive site residue, resulting in conformational perturbation. In an attempt to discriminate between these two possibilities, we determined the effects of Ado and its analogs on the rate of inactivation of the two enzymes. Kinetic studies of the enzyme protection afforded by Ado indicated that NEM was competing for the Ado binding site. However, noticeable differences in the values of dissociation constant (K_s) and protection constant (K_p) of hamster enzymes with respect to Ado provided conclusive kinetic proof that the reactive thiol group(s) of the hamster enzyme is located at a site distal from the catalytic site (Baker 1967). In contrast, in leishmanial enzyme, the functional −SH group(s) is located at the catalytic site.

Relationship between substrate inhibition and substrate protection : In an earlier publication from this laboratory it has been demonstrated that high concentrations of Ado exerts inhibitory effect on Ado phosphorylation by hamster enzyme (Bhaumik & Datta 1992). In contrast,

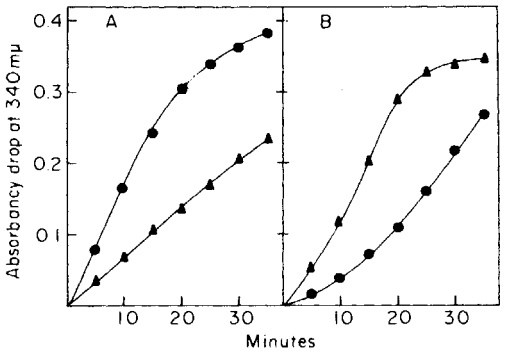

Figure 3 Progress of Ado (●) and 6-MMPR (▲) phosphorylation by adenosine kinase from leishmania (**A**) and hamster liver (**B**). Concentrations of substrates were maintained at 50 μM. Rest of the assay conditions were as described in the "Materials and Methods"

adenosine kinase from leishmanial parasite follows the classical Michaelis-Menton kinetics with increasing substrate concentration. These observations, when taken together with the findings made in the present work, seem to indicate that high concentration of Ado apparently accomplish two different things on hamster enzyme, viz. (i) enzyme inhibition and (ii) protection against NEM-mediated inactivation. In order to determine whether these two effects are interconnected, we carried out protection experiments in presence of another substrate analog (6-MMPR), which does not exert as strong substrate inhibitory effect as Ado (figure 3, Lin et al. 1988). Interestingly enough, 6-MMPR did not exert as strong protective effect on hamster enzyme. However, the same ligand, under identical condition, protected leishmanial enzyme quite efficiently. The resutls thus lead us to suggest that "protection site" might be the site which is responsible for the substrate inhibition caused by Ado.

Physiological implications : Unlike leishmanial cells, higher eukaryotic cells are sensitive to very low concentration of Ado (Fox & Kelley 1978). The toxicity of Ado in higher eukaryotes results from the inhibition of pyrimidine biosynthesis by the phosphorylated product of the adenosine kinase reaction (Ishii & Green 1973). Since, in almost all eukaryotic cells, this enzyme is abundant, a fine control of the enzyme's activity is a physiological necessity. Several lines of evidence indicate that adenosine kinase, in conjunction with adenosine deaminase, plays a significant role in regulating adenosine concentration *in vivo* (Berne et al. 1983, Plagemann 1985, Hawkins et al. 1980). However, the mechanism by which adenosine kinase regulates adenosine

Table 1 *Protection of adenosine kinase by 6-MMPR against NEM-mediated inactivation*

Enzyme Source	Experiment	Activity Remaining (%)
Parasite	Control Enzyme	100
	+ NEM (1mM)	9
	+ 6-MMPR	104
	+ 6-MMPR and NEM (1mM)	71
Hamser	Control Enzyme	100
	+ NEM (1mM)	8
	+ 6-MMPR	94
	+ 6-MMPR and NEM (1mM)	9

Enzyme (1 μM) was treated with or without NEM for 15 min at 30°C. During protection experiments, 6-MMPR at a concentration of 100 μM was added prior to the addition of NEM. Rest of the conditions were same. After the indicated period, DTT (2mM) was added. Enzyme was diluted appropriately in a buffer (10mM Tris *p*H 7.5, 20% glycerol and 1mM DTT) to dilute 6-MMPR concentration to a minimum level and assayed. [^{3}H]-Adenosine (100-250 cpm/pmol), at concentrations of 50 and 15 μM was used as substrate for assay of leishmanial and hamster enzymes respectively.

concentration remains ill understood. In light of these findings, it becomes worth at this juncture to assess the implications of our observations. The role of adenosine kinase in maintaining the intracellular concentration of adenine nucleotide, by providing a driving force for the movement of adenosine from extra-cellular environment, has been demonstrated by several workers (Schrader et al. 1972, Parks & Brown 1973, Holmsen & Rosenberg 1968). The facts that (i) adenosine kinase deficient cells excrete massive amounts of purines (Chan et al. 1973), (ii) the uptake of Ado in adenosine kinase negative cells is drastically reduced (Iovannisci & Ullman 1984) and (iii) the extents of intracellular phosphorylation of Ado is dependent upon the concentration of extracellular Ado and levels of adenosine kinase and adenosine deaminase activities (Dawicki et al. 1988), provide support to this concept.

In order to draw an interconnection between the preceeding observations and the results presented in this communication, we propose a generalized scheme for the mechanism of utilization of adenine nucleoside (figure 4). In this scheme adenosine kinase from higher eukaryotes, as opposed to enzyme from purine auxotropic leishmanial parasite, can assume two forms (E and E') depending upon the concentration of intracellular Ado. At low Ado concentrtion the enzyme (E) catalyzes the reaction by binding at its high affinity catalytic site. However, with increase in Ado concentration, the enzyme reversibly binds Ado to both catalytic and non-catalytic sites and assumes inactive form (E'). This results in reversible removal of the part of the active enzyme (E) from the reaction flux with apparent inhibition of enzyme activity. On the contrary, in leishmanial enzyme, binding of Ado takes place only through the catalytic site. Consequently, there is no excess substrate inhibition. Thus, "substrate inhibition" seems to be one of the mechanism by which higher eukaryotic cells regulate the optimum production of adenine nucleotide. However, the parasitic protozoa, because of purine auxotrophy, can not afford to have the similar kind of regulatory mechanism. This is reflected in lack of sensitivity of adenosine kinase to excessive adenosine.

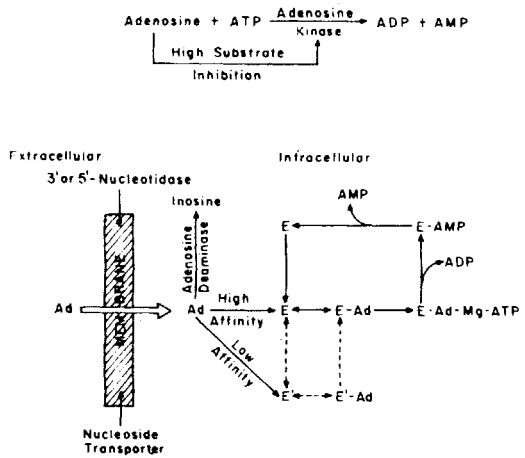

Figure 4 Model for Ado utilization by adenosine kinase. Dotted arrows indicate the alternative pathway that account for adenosine inhibition via E'

The results obtained in this study thus provide a possible physiological basis for the expression of differential catalytic behaviour of adenosine kinase from divergent sources. While our data appear to suggest a role of $-SH$ group in substrate binding/or catalysis, they do not absolutely preclude a structural function of cysteine residues. Nevertheless, it is amply clear that the functional cysteine residues in enzymes from two sources because of differential localization on the protein backbone, carry out differential functions. Additional studies related to structure-function properties of the enzymes could provide a basis for their substrate recognition properties.

Acknowledgements

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