Probing the function(s) of active-site arginine residue in *Leishmania donovani* adenosine kinase

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The presence of arginine at the active site of *Leishmania donovani* adenosine kinase was studied by chemical modification, followed by the characterization of the modified enzyme. The arginine-specific reagents phenylglyoxal (PGO), butane-2,3-dione and cyclohexane-1,2-dione all irreversibly inactivated the enzyme. In contrast, adenosine kinase from hamster liver was insensitive to these reagents. The inactivation of the enzyme by PGO followed pseudo-first-order kinetics, with a second-order rate constant of 39.2 $min^{-1} \cdot M^{-1}$. Correlation between the stoichiometry of PGO modification and extent of inactivation indicated that modification of a single residue per molecule suffices for the loss of activity. Reactivity of the essential arginine residue towards PGO was affected by the presence of adenosine (Ado) and other competing alternative substrates, consistent with an arginine residue located proximal to the Ado-binding site. The enzyme showed an intrinsic fluorescence with an emission maximum at 340 nm when excited at 295 nm. The protein fluorescence was partially quenched on addition of Ado. PGO modification also led to significant quenching of the fluorescence. However, the fluorescence of the Ado-protected enzyme, which displayed 82% of the original activity after PGO treatment, was retained. The kinetic analyses of the partially modified enzyme showed an increase in the K_m for Ado from 14 to 55 μM. Furthermore, the inability of the modified enzyme to bind to 5′-AMP–Sepharose 4B affinity column provided additional evidence that modification is attended by decrease in affinity of the enzyme for Ado. The results are consistent with the interpretation that modification of the active-site arginine residue affects activity by interfering with the binding of the substrate to the active site.

INTRODUCTION

Parasitic protozoa are the causative agents for a variety of fatal diseases. The lack of therapeutic selectivity is the major obstacle towards successful treatment of these ailments. In order to develop a rational chemotherapeutic approach, workers from various laboratories have focused attention on the purine-nucleotide-synthesizing-pathway enzymes of these parasites [1–3]. The importance of this pathway stems from the observation that all parasitic protozoa studied to date lack the ability to synthesize purines *de novo* and thus scavenge purines from the host using its own unique set of purine-salvage enzymes [4,5].

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20), one of the key enzymes of the purine-salvage pathway, catalyses the transfer of terminal phosphate from ATP to Ado [6]. Studies from several laboratories have attached significant importance to this reaction in most eukaryotic cells, and especially in these purine-auxotrophic parasitic protozoa [7,8]. Understanding the catalytic mechanism of this enzyme from *Leishmania donovani*, a parasite belonging to the family Trypanosomatidae, is the primary goal of this laboratory. These studies are important both from the fundamental point of view and in the hope that a detailed knowledge of this unique leishmanial enzyme may provide relevant information necessary towards designing selective inhibitors which could be effective in the treatment of visceral leishmaniasis ('Kala-azar'), a disease known to be caused by this organism.

A wealth of information collected over the past several years has revealed differential properties of adenosine kinase from various sources [9–12]. Despite these studies, virtually no information, at present, is known regarding the active-site structure and the molecular mechanism associated with adenosine-kinase-mediated Ado phosphorylation. This is primarily due to (i) the lack of information about the active site amino acid residues, (ii) the unavailability of a cloned gene, and (iii) the absence of an X-ray structure of a substrate (or substrate analogue) complex of adenosine kinase from any known source. Clearly the chemical-modification approach, with its direct probing of active-site structure, remains the only approach left at this juncture.

Previous studies from this laboratory demonstrated that adenosine kinase from *L. donovani* displays many different, as well as some identical, characteristics as compared with the corresponding enzyme from other known eukaryotic sources [12–14]. Chemical-modification studies and substrate-competition experiments with Ado and various other nucleoside analogues locked in defined conformations suggested the existence of two discrete catalytically active nucleoside-interacting sites on the enzyme molecule [14]. Furthermore, the parasite enzyme, unlike the adenosine kinase from higher eukaryotes, does not seem to contain any regulatory Ado-binding site [15]. Studies with selective thiol blockers have, in addition, revealed that the leishmanial enzyme harbours two reactive thiol groups at, or near, the active site [15].

In a continuing effort to elucidate the role of other active-site residues, we have carried out chemical-modification experiments with various arginine-specific modifiers [16–18]. Since the strategy of our approach is based upon probing the mechanistic aspects of adenosine kinase from higher eukaryotic and leishmanial sources, the adenosine kinase from hamster, a natural host of *L. donovani*, was included for comparative studies. The evidence presented here, for the first time, indicates the presence of one arginine residue exclusively at the Ado-binding site of the leishmanial enzyme. Furthermore, evidence is presented to sug-

Abbreviations used: PGO, phenylglyoxal; DTT, dithiothreitol; Ado, adenosine; DMSO, dimethyl sulphoxide.
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gest that modification of the arginine residue by PGO leads to impaired binding of Ado by the enzyme.

EXPERIMENTAL

Materials

Phenylglyoxal (PGO), cyclohexane-1,2-dione and butane-2,3-dione were the products of Sigma Chemical Co. [7-^{14}C]PGO (23 mCi/mmol) was purchased from Amersham Corp. PGO, at concentrations of 500 mM, was dissolved in 100% dimethyl sulphoxide (DMSO), while cyclohexane-1,2-dione and butane-2,3-dione were dissolved in water. DMSO, up to a concentration of 12–15%, did not have any effect on the enzyme activity from either source. [^{125}I]Iodide was obtained from Bhabha Atomic Research Center, Bombay, India. Sources of other products were as described previously [12–15].

Sources of enzymes

Adenosine kinase from *L. donovani* (MHOM/IN/1978/UR6) was purified to homogeneity using the method described previously [12]. The enzyme preparation was routinely checked for purity. For all the experiments, unless otherwise mentioned, an enzyme preparation from a single batch was used.

The enzyme from hamster liver was also purified according to a recently published procedure [15].

Enzyme assays and initial-velocity determinations

Assays of the adenosine kinase from both parasite and hamster liver were performed as described previously [15]. The kinetic constants were determined by standard published procedures [19].

Inactivation by arginine-specific modifiers

To exclude non-specific reactions, the inactivation of adenosine kinase at pH 7.5 was carried out by incubating the enzyme with appropriate amounts of modifiers at 25 °C [20]. The extent of inactivation was monitored by measuring the residual activity on aliquots withdrawn from the incubation mixture.

Protection constant

The protection constant (K_p) was determined by the method of Chuang and Bell [21]. The rate of inactivation of adenosine kinase by PGO, in the presence of different fixed levels of Ado, was determined under conditions where pseudo-first-order kinetics prevailed. Reaction mixtures (20 μl) at 25 °C contained 0.75 μM (28 μg/ml) enzyme, 0–300 μM Ado and 25 mM PGO in 20 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol (DTT) and 20% (v/v) glycerol. At indicated time intervals, activity was determined in appropriately diluted aliquots. From a plot of log(percentage of remaining activity) against time, the rate constants of inactivation were determined in the absence of protector and over a range of protector concentrations. K and K_0 signify rate constants in presence and absence of protecting compound.

Other determinations

Immunoblotting of the enzyme protein, using an *L. donovani*-adenosine-kinase-specific antibody, was carried out as described previously [14]. Fluorescence-quenching measurements were carried out in a fluorescence polarization spectrometer (Hitachi F4020) at room temperature.

5′-AMP–Sepharose 4B affinity chromatography

Affinity chromatography of unmodified and PGO-modified enzyme was carried out as follows. The pooled fraction containing the adenosine kinase activity, eluted from the Sephadex G-100 column [12], was divided into three equal parts. One part was used as the source of unmodified enzyme. Of the other two parts, one part was exposed to 25 mM PGO for 30 min to completely inactivate the enzyme, while PGO treatment of the other part was carried out identically, but in the presence of 1 mM Ado. All the enzyme fractions, after extensive dialysis against buffer [20 mM Tris/HCl (pH 7.5)/1 mM DTT/20% glycerol], were loaded on identically sized 5′-AMP–Sepharose 4B columns. After washing with buffer [100 mM potassium phosphate (pH 7.5)/1 mM EDTA/0.1 mM EGTA/1 mM DTT/5% glycerol/1 mM PMSF and 1 mM benzamidine hydrochloride, containing 1 M KCl], the columns were run identically with buffer [100 mM Tris/HCl (pH 9.0)/1 mM EDTA/0.1 mM EGTA/1 mM DTT/20% glycerol/1 mM PMSF/1 mM benzamidine hydrochloride, containing 1 mM Ado]. Fractions (100 μl) were collected, and the enzyme protein was detected either by activity assay or by immunoblotting.

Stoichiometry of [7-^{14}C]PGO modification

Incorporation of [7-^{14}C]PGO into the protein was monitored by the procedure of Koland et al. [22] with some modifications. Adenosine kinase (34 μg), in 20 mM Tris/HCl (pH 7.5)/1 mM DTT/20% glycerol, was incubated with 6.6 mM [7-^{14}C]PGO (23 mCi/mmol) in a final volume of 90 μl. At indicated time intervals, aliquots (16 μl) of the incubation mixture were withdrawn and added to tubes containing 90 μl of above buffer plus 100 mM unlabelled PGO. For activity measurements, aliquots (3 μl) were diluted appropriately and assayed by using a standard procedure [12]. [7-^{14}C]PGO incorporation into the remaining mixture (100 μl) was carried out after trichloroacetic acid precipitation. The precipitate formed was collected on unlabelled-PGO-soaked nitrocellulose membrane filters, washed several times with 5% trichloroacetic acid, water and 95% ethanol. The papers were dried in an oven and counted for radioactivity in a liquid-scintillation counter. An enzyme blank served as the control.

RESULTS

Reaction with PGO, butane-2,3-dione and cyclohexane-1,2-dione

Exposure of leishmanial adenosine kinase with 25 mM PGO led to progressive loss in the adenosine-phosphorylating activity at pH 7.5. In contrast, the corresponding enzyme isolated from hamster liver remained more or less unaffected in the presence of an equivalent amount of PGO (Figure 1). It must be emphasized that there was no detectable inactivation of the enzyme in mixtures containing PGO-free aliquots of DMSO solvent used to prepare PGO solution (results not shown).

Since PGO has occasionally been shown to react with residues other than arginine, the two other more selective arginine-modifying agents, butane-2,3-dione and cyclohexane-1,2-dione, were tested on both the enzymes [23,24]. The enzyme from the parasite was completely inactivated by both these reagents within a very short period (Figure 2). Furthermore, the similar inhibition kinetics of the enzyme with all the three reagents make it unlikely

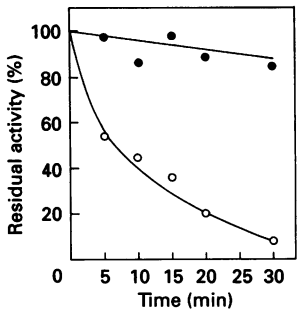

Figure 1 Effect of PGO on adenosine kinase from *L. donovani* (○) and hamster liver (●)

The enzyme from either source was incubated with freshly prepared PGO at a final concentration of 25 mM. At indicated time intervals, aliquots were removed from the incubation mixture, diluted appropriately and assayed for Ado-phosphorylating activity. The substrate concentration (50 μM) was saturating. Activities are expressed as percentages of initial activity. For details, see the Experimental section.

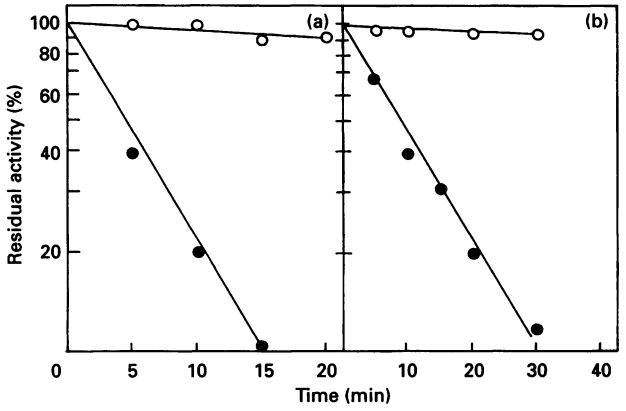

Figure 2 Effect of butane-2,3-dione (a) and cyclohexane-1,2-dione (b) on adenosine kinase from *L. donovani* (●) and hamster liver (○)

The experimental procedure was the same as in Figure 1, except that the final concentrations of butane-2,3-dione and cyclohexane-1,2-dione in the incubation mixture were maintained at 100 and 60 mM respectively.

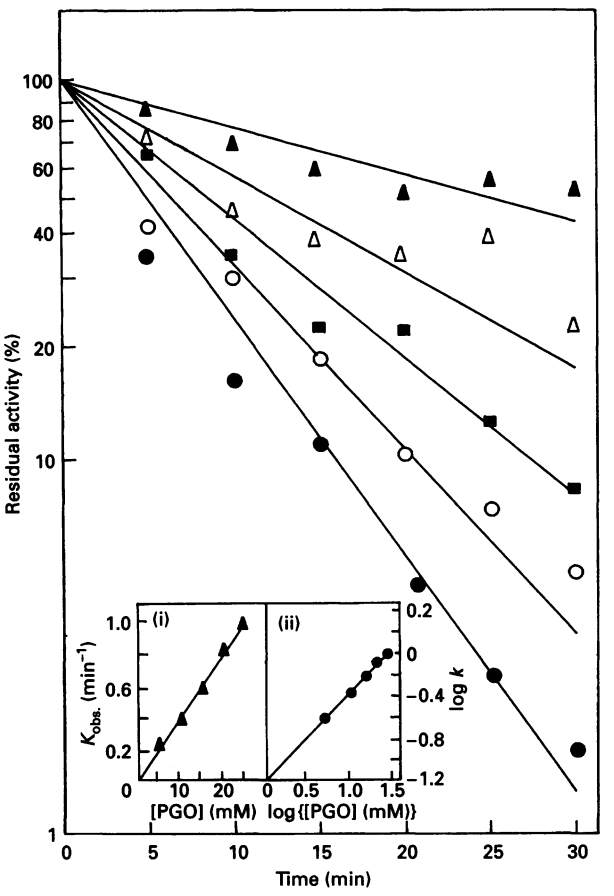

Figure 3 Kinetics of inactivation of *L. donovani* adenosine kinase by PGO

The enzyme (34 μg/ml) was incubated with various concentrations of PGO at 25 °C. The samples were removed as a function of time and assayed for activity. The PGO concentrations in various experiments were 5 (▲), 10 (△), 15 (■), 20 (○) and 25 mM (●) respectively. Inset (i) shows a plot of $K_{obs.}$ determined from the slope of the inactivation lines, as a function of PGO concentration, while inset (ii) shows a plot of the logarithm of the pseudo-first-order rate constant (k) of PGO inactivation reaction against the logarithm of the PGO concentration.

that inactivation is due to non-specific modification. The activity of the hamster enzyme, in contrast, remained almost intact even after prolonged incubation with both the reagents. The results suggest that arginine residues present in the parasite enzyme, as opposed to the enzyme from hamster, might be playing some role during the expression of the activity.

Analysis of PGO inactivation of leishmanial adenosine kinase

The inactivation of leishmanial enzyme by PGO followed pseudo-first-order kinetics (Figure 3). A plot of $k_{obs.}$ as function of PGO concentration showed no indication of saturation, implying that the reagent was not acting as an affinity analogue, but was simply attacking the native arginine residues in a bimolecular fashion (Figure 3a, inset). The slope of this secondary plot gave the second-order rate constant of 39.2 $min^{-1} \cdot M^{-1}$ for inactivation. A plot of log$k$ against log[PGO] resulted in a straight line with a slope of 0.98, thus indicating a stoichiometric binding of one molecule of PGO to one molecule of protein with concomitant loss of activity (Figure 3b, inset) [25].

Stoichiometry of PGO inactivation

The relationship between enzyme inactivation and arginine modification was determined as follows. Adenosine kinase (9–10 μM) was incubated with [7-^{14}C]PGO (6.6 mM) and assayed in parallel for both loss of activity and incorporation of [7-^{14}C]PGO. Figure 4 shows that, in the absence of Ado, the enzyme rapidly incorporated PGO with a concomitant loss in activity. At the onset of the modification reaction, the extents of inactivation observed correlated directly with the incorporation of [7-^{14}C]PGO. However, with the progress of the reaction, the extent of inactivation tended to level off. In contrast, the rate of [7-^{14}C]PGO incorporation into the protein continued to follow almost linear kinetics up to 80 min. Overnight incubation of the enzyme led to incorporation of 7 mol of [7-^{14}C]PGO/mol of enzyme, with complete loss of activity (results not shown). Extrapolation of the initial inactivation line to zero residual activity yielded a value of approx. 1, indicating incorporation of 1 mol of [7-^{14}C]PGO/mol of adenosine kinase. Inclusion of Ado

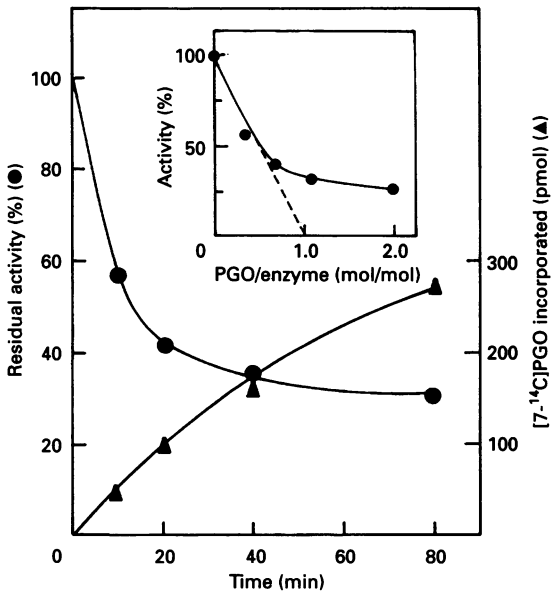

Figure 4 Correlation between inactivation of *L. donovani* adenosine kinase and incorporation of [7-^{14}C]PGO

Adenosine kinase (10 μM) was incubated with 6.6 mM [7-^{14}C]PGO for the indicated periods of time. At indicated intervals, aliquots of the incubation mixture were withdrawn and assayed in parallel for residual activity and the extent of [7-^{14}C]PGO incorporation into the protein. The M_r of the protein was taken as 38000 [12]. The inset shows a plot of the remaining activity versus incorporation at each time point. The details of the incubation, termination of reaction and analysis of arginine modification are described in the Experimental section.

during the first 30 min of modification reaction led to protection of total activity, with no incorporation of [7-^{14}C]PGO. However, with the increase in the modification time there was gradual incorporation of [7-^{14}C]PGO (results not shown). Exposure of the Ado protected and unprotected enzyme to [7-^{14}C]PGO for 4 h resulted in the incorporation of 0.68 and 2.03 mol of PGO/mol of enzyme with the retention of 85 and 4% of the original activity respectively. Clearly the presence of Ado caused incorporation of [7-^{14}C]PGO into the nonessential residues.

Irreversibility of PGO inhibition

In an initial report it was claimed that PGO inactivation of enzymes could be reversed at pH 7–7.5 [16]. In view of this we checked our inactivation results at pH 7.5. Complete removal of PGO from inactivation mixture did not result in recovery of the activity. In fact prolonged dialysis led to further inactivation. Irreversible modification of a number of enzymes by PGO has also been reported [26–28].

Protection against inactivation

In an attempt to localize the site of the reactive arginine residue, protection of the enzyme activity by substrate was studied. Various ligands were tested for their ability to protect the parasite enzyme against inactivation by PGO, butane-2,3-dione and cyclohexane-1,2-dione (Table 1). Compounds which are competitive inhibitors of Ado phosphorylation and alternative substrates, also afforded protection to the enzyme [13]. The extents of protection could be approximately correlated with their respective affinities for the enzyme. In contrast, formycin A, which was shown to be an excellent substrate for leishmanial

Table 1 Protection of *L. donovani* adenosine kinase against inactivation by PGO, butane-2,3-dione (BDO) and cyclohexane-1,2-dione (CHD)

The enzyme, in presence of the above compounds (1 mM), was exposed separately to PGO (25 mM), butane-2,3-dione (100 mM) or cyclohexane-1,2-dione (60 mM) for 30 min. The aliquots were withdrawn, diluted appropriately and assayed for Ado-phosphorylating activity (results have an error of $\pm 5\%$, $n = 5$). The inhibition constants of ligands, which are alternative substrate and/or competitive inhibitors of Ado phosphorylation, are indicated. Formycin A, although a substrate, is a non-competitive inhibitor of Ado phosphorylation. Deoxyadenosine, 2-chloroadenosine (2-cl.Ado) and formycin B are neither substrates nor inhibitors [14]. Abbreviation: 6-MMPR, 6-methylmercaptopurine riboside.

Experiment	Activity (%) PGO	BDO	CHD	K_s/K_i (μM) (Ado phosphorylation)
PGO	1.5	–	–	–
BDO	–	7	–	–
CHD	–	–	5	–
Ado	100	100	60	2.7
dAdo	3	8	5	–
2-cl.Ado	4	9	5	–
6-MMPR	15	33	63	50
Tubercidin	23	52	80	42
Formycin A	2	8	6	–
Formycin B	3	8	5	–
AMP	48	79	50	48

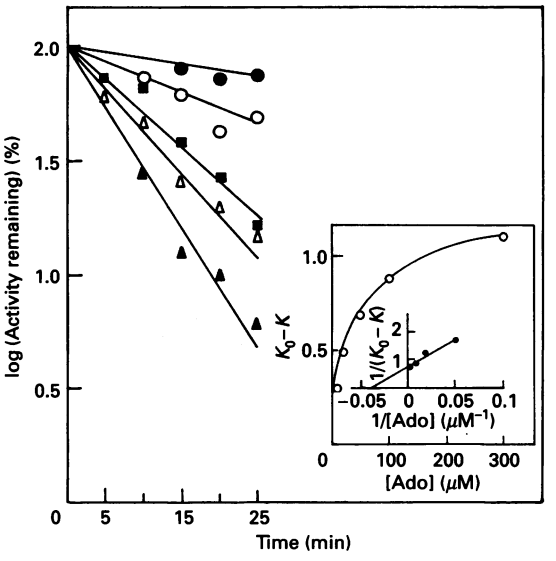

Figure 5 Quantitative evaluation of K_s of enzyme-adenosine complex against PGO-mediated inactivation

Incubation of the enzyme with 25 mM PGO was carried out with Ado concentrations of 0 (▲), 10 (△), 50 (■), 100 (○) and 300 μM (●). The results in the inset are presented in the form of Lineweaver–Burk and Michaelis plots.

adenosine kinase but a non-competitive inhibitor of Ado phosphorylation [14], did not confer any protection from PGO-mediated inactivation. ATP and ADP, which did not have much protective effect on the PGO and cyclohexane-1,2-dione-mediated inactivation showed some protection on butane-2,3-dione-mediated inactivation (results not shown). The reason for this is not clear at present. It must be mentioned that, although the steady-state kinetic analysis could not be applied to assess the

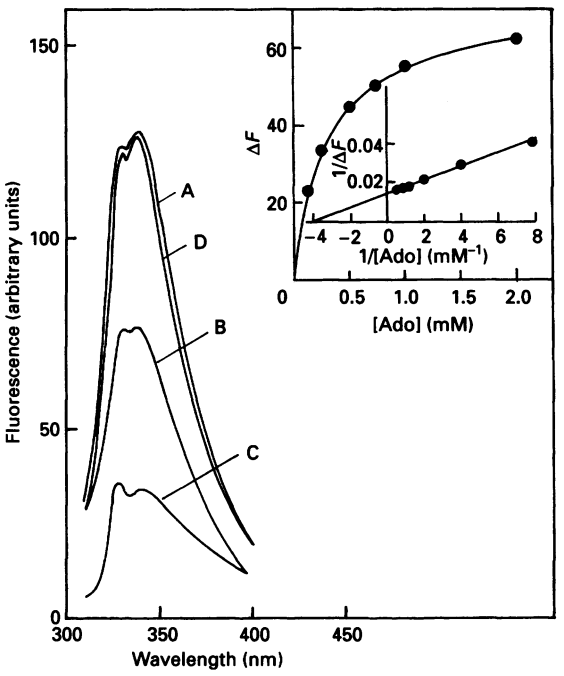

Figure 6 Fluorescence emission spectra of the native (a), Ado (750 μM)-exposed (b), PGO-treated (c) and Ado-protected, but PGO-treated (d), enzyme

Identical aliquots of enzyme (80 μg/ml) were treated with 10 mM PGO for 30 min and immediately passed through spun (Sephadex G-25) columns. After extensive dialysis of the enzyme fractions, the fluorescence measurements were carried out. Under these conditions Ado-protected enzyme (**d**) retained 85% of the original activity, while unprotected enzyme (**c**) lost almost 82% of the original activity. The inset shows Ado-dependent quenching of the enzyme fluorescence. ΔF denotes fluorescence change at various concentrations of Ado.

effects of these modifiers, the effectivity of these reagents is dependent upon their respective affinities for the enzyme.

The phenomenon of enzyme protection by Ado thus provided us with a method to determine the dissociation constant (K_p) of the enzyme–protector complex using the expression derived by Scrutton and Utter [29] and Chuang and Bell [21]. Rate constants were determined for inactivation of the enzyme in presence of 25 mM PGO at a series of Ado concentrations. The plot of $1/K_o - k$ against 1/Ado was linear and yielded a value of 20.8 μM (Figure 5, inset). The value closely corresponded with the K_m for Ado and hence supports the assignment of arginine at the active site.

Fluorescence properties of the modified enzyme

When excited at 295 nm, the enzyme exhibited a fluorescence with an emission maxima at 340 nm, characteristic of a tryptophan-containing protein (Figure 6). Upon addition of Ado, the intrinsic fluorescence was partially quenched. The extent of quenching was found to be dependent on the concentration of Ado and was saturable (Figure 6, inset). Treatment of the enzyme with PGO also caused a significant reduction in the fluorescence. The fluorescence of the PGO-modified enzyme was not changed by the addition of Ado. In contrast, an identical aliquot of the Ado-protected enzyme fraction which retained 82% of the activity did not display any significant reduction in fluorescence. It must be mentioned that the fluorescence-emitting property of butane-2,3-dione-treated enzyme was similar to that of PGO-modified enzyme (results not shown).

These findings are consistent with the interpretation that at least one tryptophan residue occurs at or near the Ado-binding site. The appearance of two peaks in the fluorescence spectra might be due to two tryptophan residues. This is under further investigation.

Reduced interaction of Ado with the modified enzyme

Inhibition of enzyme activity by PGO can be either due to lack of substrate binding or interference at the catalytic step. Determination of the K_m for Ado of the PGO-modified enzyme gave a value of 50 ± 5 μM compared with 14.3 ± 2 μM for the unmodified enzyme (Table 2). Moreover, the comparison of the specificity constants ($V_{max.}$ and $K_{cat.}/K_m$) for the modified and unmodified enzyme indicated that the effect was indeed at the substrate-binding step.

The reduced affinity of the modified enzyme towards Ado is obviously due to alteration in the binding energy. For enzyme–substrate interactions, the incremental binding energy is given by [30]:

$$RT\ln[(K_{cat.}/K_m)'/(K_{cat.}/K_m)]$$

where $(K_{cat.}/K_m)$ and $(K_{cat.}/K_m)'$ are the specificity constants for the reactions of enzyme and modified enzyme with substrate determined from Michaelis–Menten equation. The effect of modification on the empirical binding energy thus indicated the possibility for loss of uncharged hydrogen bond in the transition-state complex in the modified enzyme [30].

Elution behaviour of the modified enzyme from the 5′-AMP–Sepharose 4B column

The inefficient binding of Ado to the inactivated enzyme was further examined directly. In this experiment the specific property of adenosine kinase binding to 5′-AMP–Sepharose 4B column was utilized to study the differential binding behaviour of the native enzyme from that of the PGO-modified enzyme [12]. Since 5′-AMP is a competitive inhibitor of Ado [13,14], the inability of the enzyme to bind to 5′-AMP–Sepharose 4B column can be regarded as the loss of Ado-binding capacity. The results presented in Figure 7 show that indeed this was the case. The unmodified enzyme interacted with the 5′-AMP–Sepharose column in the usual manner and was eluted with Ado-containing buffer (Figure 7a).

In contrast the PGO-modified inactive enzyme, under identical conditions, came out in the flowthrough fraction and was detected by Western-blot analysis (Figure 7b). From the experiment described in Figure 7(b) one can argue that complete inactivation resulted from non-selective modification of the enzyme and hence loss of Ado/AMP-binding capacity. In order to rule out such a possibility, another identical aliquot of the enzyme, which was protected to the extent of 80% by Ado during PGO treatment, was analysed. Interestingly, the protected portion of the enzyme behaved like the unmodified enzyme, while the inactivated fraction came out in the flowthrough fraction (Figure 7c). Determination of K_m for the Ado-protected activity yielded a value (14 μM) similar to that of the unmodified enzyme. Moreover, the 80%-protected enzyme displayed increased sensitivity to PGO modification, indicating essentiality of the active-site arginine residue (results not shown). Western-blot analysis of the flowthrough and Ado-eluted fractions indicated that the respective amounts of protein distributed in the two fractions

Table 2 Kinetic parameters of *L. donovani* adenosine kinase after modification with PGO

Experiment	K_m (μM)	$V_{max.}$ (μmol/min per mg)	K_{cat}/K_m (s^{-1} μM^{-1})	ΔG† (kJ/mol)
Enzyme	14.3±2	20	0.9	−33.95
Enzyme + PGO*	50±3	10	0.126	−28.96

* The enzyme was treated with 10 mM PGO for 10 min to cause approx. 50% inactivation.
† $\Delta G = -RT\ln(K_{cat}/K_m)$.

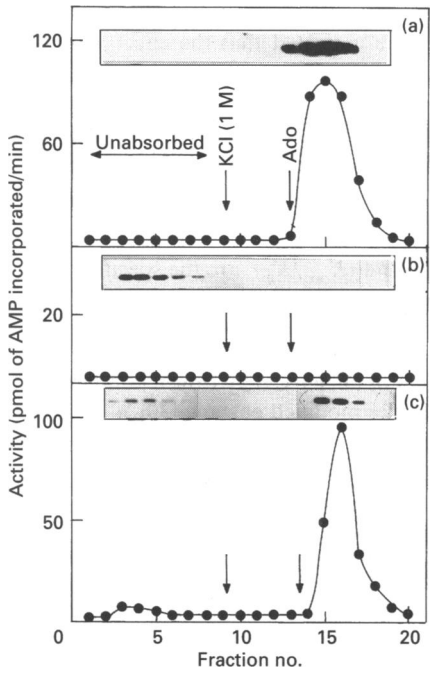

Figure 7 Elution behaviour of the native (a), PGO-modified (b) and Ado-protected, but PGO-exposed (c), enzyme from 5′-AMP–Sepharose 4B column

The details of the procedure are given in the Experimental section. The inset shows the Western-blot analysis of the corresponding samples eluted from the column.

correlated well with the extents of modification as determined by activity protection assay.

DISCUSSION

In an effort to (i) understand the catalytic mechanism of the parasite enzyme, (ii) study the possible common and distinguishing features of its active site from other known adenosine kinases and (iii) explore the possible role of different amino acid residues which are critically connected with the functional activity of the enzyme, we have embarked on a plan to map the active-site domain of the leishmanial adenosine kinase.

Chemical-modification studies and kinetic experiments, presented here for the first time, provide an evidence that PGO, butane-2,3-dione and cyclohexane-1,2-dione, known to be selective arginine-modifying agents [31], inhibit adenosine kinase of *L. donovani* (Figures 1 and 2). In contrast, the corresponding enzyme from the hamster liver remained mostly unaffected by these reagents. Treatment of *L. donovani* adenosine kinase with the above three reagents showed pseudo-first-order inhibition kinetics. Furthermore, a direct proportionality between log$k$ and log[PGO], even at very high concentrations of inhibitor, is consistent with inactivation occurring concomitantly with modification. The lack of correlation between the rate of inactivation of the enzyme and incorporation of [7-^{14}C]PGO at the later stages of modification was due to incorporation of PGO into the non-essential residues (Figure 4). Moreover, the differences in the nature of the inactivation curves presented in Figures 1 and 4 can be attributed to the differences in the concentrations of PGO used in respective experiments.

The protection studies, carried out with Ado and other substrate analogues, are consistent with the view that arginine residue is at or near the active site of the enzyme. The finding that K_p for Ado (20.8 μM), based on protection of the kinase against modification, is similar to K_m for Ado, provides added evidence in favour of the reactive arginine residue being located within the Ado-binding domain of the catalytic site. Kinetic analyses with native and partially modified enzyme showed an increase in K_m (from 14 to 55 μM) value with marked alteration of $V_{max.}$ or $K_{cat.}/K_m$, indicating a reduced affinity of the enzyme for Ado. Furthermore, a higher K_m value of the partially modified enzyme, with simultaneous change of $V_{max.}$, is consistent with the fact that inactivation was not due to modification of a fractional population of enzyme molecule [32]. The effect of modification on the activation energy ($\Delta\Delta G = 4.99$ KJ/mol) could also be characteristic of the deletion of an uncharged hydrogen bond in the transition-state complex in the modified enzyme [30].

Tryptophan-fluorescence-quenching studies and activity-measurement experiments led us to make the following inferences: (i) the Ado-binding pocket of the enzyme contains at least one tryptophan residue located proximal to the essential arginine residue; (ii) positioning of the tryptophan residue(s) within the active-site domain makes its fluorescence emission property amenable to alteration by agents/ligands which have affinity for the site; and (iii) alternatively, the loss of fluorescence observed following PGO or Ado treatment may be attributed to conformational tightening of the active site. Thus the modification could simply cause interference with substrate binding by blocking entry, just as substrate could block entry of the modifying agent. The large difference of K_m (14 μM), determined kinetically, from the K_d (250 μM) estimated from fluorescence-titration experiment may have several possible explanations: (i) synergism might exist for Ado binding in the presence of ATP and Mg^{2+} during the catalytic reaction; (ii) no direct role for tryptophan in substrate binding or catalysis has yet been obtained; and (iii) the proximity of the tryptophan residue with the active-site arginine remains to be determined. Furthermore, the existence of additional Ado-binding site cannot also be ruled out. Clearly, site-directed protein-mutagenesis studies are needed to look into the possibilities.

In a number of cases an arginine residue is thought to serve as a cationic site for binding of a negatively charged group of substrate or coenzyme [33–36]. Several examples are also known in which an arginine residue is apparently involved in catalysis [37–39]. Bennett et al. [40], using adenosine kinase from H.Ep.2 cells, demonstrated that the ionization of the N-1 proton of purine nucleosides/analogues plays a crucial role in determining the substrate specificity/rate of phosphorylation. In view of this finding, it is possible that the reactive arginine residue discovered

in this study may also participate in the whole process by directing/reorienting the substrate to the active site for subsequent reaction. Notwithstanding these difficulties, the present finding that modification of a specific arginine, one of the constellation of active-site residues of leishmanial adenosine kinase and not hamster adenosine kinase, alters substrate binding lead to the speculation that probably the 'arginine function' of the leishmanial enzyme is carried out by another structure of hamster enzyme. A rational explanation of this finding might open up new avenues towards understanding the differential behaviour of adenosine kinase from divergent sources and will enrich our knowledge, hitherto unknown, of adenosine kinase enzymology in general.

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