Two conformationally vicinal thiols at the active site of *Leishmania donovani* adenosine kinase

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Inactivation of adenosine kinase (Adk) from *Leishmania donovani* correlates with the modification of two conformationally vicinal cysteine residues. In contrast, Adk from hamster liver, despite being sensitive to monothiol-blocking reagents, was insensitive to dithiol modifiers. Inactivation kinetics and substrate-protection studies along with double-modification experiments successively with *N*-ethylmaleimide in the presence of Ado and sodium *m*-arsenite–2,3-dimercaptopropanol or diazenedicarboxylic acid bis-*N,N'*-dimethylamide supported assignment of the two thiols at the Ado-binding site. Cystine bridge formation impaired the ability of the modified enzyme to bind to the substrate. Tryptophan fluorescence of the enzyme was quenched after modification by dithiol-blocking reagents with concomitant loss of activity. However, treatment of the enzyme with methyl-

methanethiosulphonate (MMTS) led to complete inactivation without a marked change in protein fluorescence. Ado protected both fluorescence and catalytic activity against inactivation by both MMTS and dithiol-blocking reagents. Stern–Volmer quenching analysis of the native and Ado-complexed enzyme suggested that, of the four tryptophan residues, at least one is located at or near the active site. Furthermore quenching constants of native, Ado-complexed and dithiol-modified enzyme in the presence of either acrylamide or KI indicated spatial proximity of tryptophan and two cysteine residues within the hydrophobic domain of the Ado-binding site. Taken together the results suggest important function(s) for the cysteine residue(s). A schematic model is proposed to explain the inactivation of the enzyme by both monothiol- and dithiol-blocking reagents.

INTRODUCTION

The development of a chemotherapeutic regimen against visceral leishmaniasis, a devastating disease caused by the purine auxotrophic parasitic protozoan *Leishmania donovani*, is often hampered because of the appearance of drug resistance and the similarity of its biochemical pathways to those of the host [1,2]. However, the inability of these parasites to synthesize purine bases *de novo* has allowed investigators to assess the chemotherapeutic potential of their unique purine-salvage-pathway enzymes and other plausible drug-targeting sites [3–5].

In order to assess the prospects of *L. donovani* adenosine kinase (Adk) being one such drug-targeting or activating enzyme, detailed analysis of it is being undertaken in this laboratory [6–8]. The rationale for this notion stems from the following observations: first, in most mammalian cells, the AMP pool constitutes the major source of host purines [9]; second, mutant cells of *L. donovani*, lacking Adk, incorporate Ado to a much lesser extent than its wild-type [10]; finally, morphological transformation of the parasite from the promastigote (extracellular) to the amastigote (intracellular) stage is accompanied by a concomitant increase in Adk activity and thus Adk could be considered to be a developmentally regulated stage-specific peak-function enzyme [11,12]. Understanding the catalytic mechanism of this enzyme thus becomes an obvious necessity.

Information accumulated during the past decade has indicated differential properties of Adk from divergent sources [13–16]. However, very little effort so far has been spent in elucidating its mechanism of action or differential substrate-utilizing properties.

Also unknown is the amino acid composition of its active site. X-ray-structural data on a substrate (or substrate analogue) complex, which have the potential for providing information critical for the design or improvement of analogues, are also non-existent due to the lack of an available cloned gene or appreciable amounts of purified enzyme. In view of these shortcomings, this laboratory initiated work on the analysis of the active-site domain of the enzyme isolated from *L. donovani*. Recently, it has been reported that the parasite enzyme, in contrast with its counterpart host enzyme, contains a single arginine residue at or near the active site [17]. In this paper, we report that the active site of the enzyme harbours two reactive conformationally vicinal thiols. Furthermore, spectroscopic studies allowed us to determine the environment of the active site.

EXPERIMENTAL

Materials

N-Ethylmaleimide (NEM), methylmethanethiosulphonate (MMTS), sodium *m*-arsenite (arsenite), 2,3-dimercaptopropanol (BAL) and diazenedicarboxylic acid bis-*N,N'*-dimethylamide (diamide) were from Sigma Chemical Co. All the reagents were freshly dissolved in deionized water. Concentrations of NEM and diamide were determined by using their respective absorption coefficients: that for NEM at 305 nm is 620 M⁻¹·cm⁻¹ and that for diamide at 296 nm is 3000 M⁻¹·cm⁻¹. Arsenite–BAL was prepared by mixing arsenite and BAL solutions in a 1:1

Abbreviations used: Ado, adenosine; DTT, dithiothreitol; MMTS, methylmethanethiosulphonate; BAL, 2,3-dimercaptopropanol; NEM, *N*-ethylmaleimide; arsenite, sodium *m*-arsenite; diamide, diazenedicarboxylic acid bis-*N,N'*-dimethylamide; Adk, adenosine kinase; Triostam, trivalent sodium antimonyl gluconate.

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molar ratio. To avoid precipitation, the concentration was not allowed to exceed 500 μM . Sources of other materials were as described previously [17].

Source of enzyme

Adk from *L. donovani* (MHOM/IN/1978/UR6) was purified to homogeneity [6]. The monomeric enzyme, routinely checked for purity, displayed a single polypeptide of molecular mass about 38 kDa on SDS/PAGE. For all the experiments, unless otherwise mentioned, an enzyme preparation from a single batch was used. Before chemical modification studies, the enzyme was extensively dialysed against buffer containing 20 mM Tris/HCl, pH 7.5, and 5% glycerol.

Enzyme assays and initial-velocity determinations

Assays of the enzyme were carried out by a previously described procedure [6] except that in all the assays, unless otherwise mentioned, DTT was omitted.

The kinetic constants were determined using standard published procedures [8,17].

Inactivation by -SH group modifiers

The modification of Adk at pH 7.5 was carried out by incubating the enzyme (0.6–0.8 μM) with appropriate amounts of modifier at 25–30 °C. The extent of inactivation was monitored by measuring the residual activity in aliquots withdrawn from the incubation mixture. Unlike the observation of Cunningham and Fairlamb [5], dilution of the aliquots did not result in reactivation of the enzyme. All the modification experiments were carried out in triplicate and repeated several times over a period of at least 2 years.

Protection constant

The protection constant (K_p) was determined using the method of Chuang and Bell [18]. The rate of inactivation of Adk by arsenite-BAL, in the presence of different fixed levels of Ado, was determined under conditions in which pseudo-first-order reaction kinetics prevailed. The reaction mixture (12 μl) at 25 °C contained 0.40 μM (15 $\mu\text{g}/\text{ml}$) enzyme, 0–8 μM Ado, 160 μM arsenite-BAL, 20% glycerol and 1 mg/ml BSA. 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 the presence and absence of protecting compound.

5'-AMP-Sepharose 4B affinity chromatography

Affinity chromatography of unmodified and SH-modified enzyme was carried out as follows. Purified enzyme was divided into several portions: one served as the control and the others were subjected to modification by arsenite-BAL or MMTS for 60 min (for details refer to the Figure legends). The concentration of the blocker was maintained between 100 and 150 μM . After treatment, the samples were dialysed extensively against buffer (20 mM Tris/HCl, pH 7.5, and 20% glycerol) and loaded on identically sized 5'-AMP-Sepharose 4B columns. After washing with buffer (containing 100 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine hydrochloride and 5% glycerol containing 1 M KCl), the

columns were run identically with buffer (consisting of 100 mM Tris/HCl, pH 9.0, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine hydrochloride and 20% glycerol containing 1 mM Ado). Fractions (100 μl) were collected and the protein was detected by either activity assay (in the presence or absence of 20 mM DTT) or immunoblotting using anti-Adk IgG as the probe [8].

Fluorescence measurements

Unless otherwise stated, fluorescence measurements of the enzyme (0.8 μM) in 20 mM Tris/HCl (pH 7.5)/10% glycerol were made using an Hitachi F4020 spectrofluorimeter. After excitation at 295 nm, emission was monitored between 300 and 400 nm. The slits for both excitation and emission were set at 5 nm. The average values for three 5 s interval measurements were taken using appropriate blanks. The correction for inner filter effect ($A_{295} > 0.1$) was calculated using the formula:

$$F = F_{\text{obs}} \text{ antilog } [(A_{\text{exc}} + A_{\text{em}})/2] \times \text{dilution factor}$$

where A_{exc} and A_{em} are absorbance at excitation and emission wavelengths respectively [19].

Quenching experiments were carried out using acrylamide and KI. In order to maintain constant ionic strength and to prevent I_3^- formation, a 3 M solution of KI was made in 1 M KCl containing trace amounts of sodium thiosulphate [20]. Acrylamide (3 M) was made up in water. The activity of the enzyme remained unaffected even at the highest concentrations of quencher used.

Based on the nature of the plot of F_0/F versus Q , it was assessed that the mode of quenching was collisional with heterogeneity [21], thus reducing the original Stern–Volmer equation to:

$$F_0/F = 1 + K_{\text{sv}}[Q] \quad (1)$$

The quenching parameters were determined from the modified Stern–Volmer equation as proposed by Lehrer [22]:

$$F_0/\Delta F = 1/f_a + (1/f_a K_{\text{sv}})(1/[Q]) \quad (2)$$

where F_0 and F are fluorescence in the absence and presence of quenchers respectively, $\Delta F = F_0 - F$, $[Q]$ is the concentration of quenchers, f_a is the fraction of total emission accessible to the quencher and K_{sv} is the Stern–Volmer constant.

Additional procedures

SDS/PAGE (10% gel) was performed as described by Laemmli [23]. 2-Mercaptoethanol was omitted wherever indicated. The tryptophan content of the native Adk (0.8–1.0 μM), in the presence of 6M guanidinium chloride and 30 mM DTT, was determined by a published procedure [24]. In brief, a fluorescence calibration was performed with increasing free tryptophan concentrations, and a correlation between the fluorescence of standard protein tryptophan residues, unknown protein and free tryptophan was established. The results indicated that leishmanial Adk contained 3.7 ± 0.3 mol of tryptophan/mol of protein.

RESULTS

Inactivation of *L. donovani* Adk by dithiol modifiers

In previous studies, exposure of Adk from *L. donovani* and hamster liver to monothiol-modifying reagents led to complete inactivation of the enzymes [25,26]. Since the loss of *L. donovani*

Figure 1 Effects of arsenite-BAL (●) and diamide (○) on Adk activity and re-activation of the activity by DTT

Concentrations of arsenite/BAL and diamide were maintained at 160 μM and 1 mM respectively. Control experiments without modifier (Δ) or with 300 μM arsenite ($\square$) or 150 μM BAL ($\blacksquare$) are also depicted. ———, Effect of arsenite-BAL on Adk from hamster liver. Enzyme concentration was maintained at around 0.6 μM . For details refer to the Experimental section.

Adk activity observed on exposure to NEM correlated directly with the incorporation of 2 equiv. of NEM/enzyme molecule, it was of interest to analyse the function and location of the two SH-groups on the native protein molecule. A wide variety of selective dithiol-modifying reagents are available. These reagents, differing in shape, charge, hydrophobicity and bulk, are fairly specific for vicinal thiols. Arsenite-BAL and diamide are among such compounds [27,28]. Arsenite-BAL at low concentration rapidly inactivated the *L. donovani* enzyme (Figure 1). However, the corresponding enzyme from hamster liver remained unaffected under such conditions. On addition of DTT, most (> 90%) of the dithiol-modified *L. donovani* enzyme was re-activated. Interestingly, arsenite itself had no significant effect on the enzyme activity. BAL was also ineffective in this respect. Cunningham and Fairlamb [5] reported that triostam-induced inactivation of trypanothione reductase activity of *L. donovani* was reversed after inhibitor dilution. However, we failed to observe any such reversal of kinase activity after diamide or arsenite-BAL dilution. This could reflect a differential mode of action of two structurally different modifiers on two different enzymes. It was previously proposed that the arsenite-BAL complex neutralizes the charge on arsenite thereby allowing it to penetrate the hydrophobic barrier [29].

Intramolecular localization of the disulphide bond

Do the thiol residues involved in cysteine bridge formation come from two different molecules? In order to answer this, the diamide-treated enzyme, in the presence or absence of 2-mercaptoethanol, was subjected to PAGE. The Coomassie-Blue-stained gel showed that the treated enzyme in both the presence and absence of 2-mercaptoethanol had identical mobility with that of the native enzyme, indicating the absence of dimer/multimer formation between two or more molecules (results not shown).

Figure 2 Kinetics of inactivation of *L. donovani* Adk by arsenite-BAL

The enzyme (0.7 μM) was incubated with various concentrations of arsenite-BAL at 25 °C. The samples were removed at the indicated times, diluted appropriately and assayed in the absence of DTT. The arsenite-BAL concentrations in various experiments were 25 ($\blacksquare$), 30 ($\square$), 35 ($\blacktriangledown$), 45 ($\triangle$), 50 ($\blacktriangle$), 75 ($\circ$) and 100 ($\bullet$) μM . Inset (a) shows a plot of k_{obs} determined from the slope of the inactivation lines as a function of arsenite-BAL concentration whereas inset (b) shows a plot of the logarithm of the pseudo-first-order rate constant (k) of the arsenite-BAL inactivation reactions against the logarithm of arsenite-BAL concentration.

Analysis of inactivation kinetics

The loss of catalytic activity was pseudo-first-order with respect to arsenite-BAL (Figure 2). A plot of k_{obs} as a function of arsenite-BAL concentration showed no indication of saturation, implying that the reagent was not acting as an affinity analogue, but simply stemmed from a bimolecular reaction between arsenite-BAL and the cysteine residues of the enzyme. The slope of the secondary plot gave the second-order rate constant for the inactivation of 3.75 $\text{min}^{-1} \cdot \text{mM}^{-1}$. The results of the experiment were also plotted as $\log k$ versus $\log [\text{arsenite-BAL}]$ by the method of Levy et al. [30]. The resulting plot is linear with a slope of 1.4, thus indicating that approximately one molecule of arsenite-BAL reacts with two vicinally located thiols as inactivation occurs (Figure 2, inset).

Protection against inactivation

Enhanced solvent accessibility of activated side chains of amino acids in the active sites often makes these residues more reactive to chemical modifiers than those located elsewhere in the enzyme. In an attempt to localize the positions of the two vicinal thiols, protection of the enzyme activity by substrate was studied. Various ligands, when tested for their ability to protect the enzyme against inactivation by arsenite-BAL, afforded differential protective effects (Table 1). ATP and ADP were ineffective in this respect. It should be noted that steady-state kinetic analysis is not totally applicable in chemical modification studies of these types. Therefore the effects of these reagents in the presence of protecting ligands is often correlated with the specific affinities of the ligands for the enzymes.

For enzymes that undergo either spontaneous or chemically induced inactivation and are protected against such inactivation

Table 1 Protection of *L. donovani* Adk against inactivation by arsenite–BAL

The enzyme in the presence (500 μ M) or absence of the indicated ligands was exposed to arsenite–BAL (150 μ M) for 30 min. Aliquots were withdrawn, diluted appropriately and assayed for Ado-phosphorylating activity (results have an error of $\pm 5\%$, $n = 3$). The inhibitor constants of ligands, which are alternative substrates and/or competitive inhibitors of Ado phosphorylation, are indicated. Deoxyadenosine and 2-chloroadenosine are neither substrates nor inhibitors [8]. The K_s/K_i results are taken from Ghosh and Datta [17]. Abbreviation: MMPP, methylmercaptopyrine riboside.

Experiment	Activity remaining (%)	K_s or K_i (μ M) for Ado phosphorylation
Arsenite–BAL	8	—
+ Ado	92	2.7
+ DeoxyAdo	12	—
+ 2Cl-Ado	37	—
+ Tubercidin	81	42
+ 6-MMPP	76	50
+ ATP	6	—
+ ATP–Mg ²⁺	11	—
+ AMP	72	48

Figure 3 Protection of Adk activity by Ado against arsenite–BAL-mediated inactivation

Ado at concentrations of 0 (●), 1 (□), 2 (▲), 4 (■), 6 (○) and 8 (▼) μ M was used as the protective ligand. The concentration of arsenite–BAL was fixed at 160 μ M for each incubation. At the indicated times, aliquots were removed, appropriately diluted and assayed for remaining activity. The determination of K_p for Ado from the primary data is shown in the inset.

by substrates or their analogues, a protection constant (K_p) similar to K_m can be calculated for the enzyme–substrate complex [31,32]. To ascertain the site of thiol protection against modification, rate constants were measured for the inactivation of the enzyme in the presence of 160 μ M arsenite–BAL at a series of Ado concentrations over the range of 0–8 μ M (Figure 3). The results show an Ado concentration-dependent protection of enzyme against inactivation by arsenite–BAL. The plot of $1/(k_0-k)$ against $1/[Ado]$ was linear and yielded a value for K_p^{Ado} of $3 \pm 0.5 \mu$ M (inset to Figure 3). The similarity of this value to the dissociation constant ($K_s^{Ado} = 2.7 \mu$ M) of the enzyme–

Figure 4 Sequential treatment of Adk by NEM and dithiol modifiers

Ado-protected enzyme was first exposed to NEM in order to allow the non-essential cysteine residues to be modified. After a period of 20 min, as determined by complete loss of activity in a control lacking Ado, the experimental reaction mixture was dialysed extensively to remove Ado and NEM. The enzyme was further treated with 160 μ M arsenite–BAL (○) and 1 mM diamide (●) for the indicated times and assayed as described previously. At the indicated time, DTT was added and the enzyme assayed for regeneration of activity.

Figure 5 Elution behaviour of native (A), arsenite–BAL-modified (B), Ado-protected arsenite–BAL-modified (C) and arsenite–BAL-modified and DTT-treated (D) enzyme from 5'-AMP-Sepharose 4B columns

Column eluates were assayed in the presence (○) or absence (△) of 20 mM DTT. Insets show Western-blot analysis of each fraction using anti-Adk-specific anti-IgG.

Table 2 Activity–fluorescence relationship after modification by arsenite–BAL and MMTS

The enzyme (0.6 μ M) was treated, as indicated, with arsenite–BAL (160 μ M) and MMTS (100 μ M) for 1 h. After modification, the enzyme was further exposed to DTT (20 mM) for 50 min. For the protection experiment, Ado (500 μ M) was added before modification by arsenite–BAL or MMTS. Enzyme was diluted appropriately during the assay to reduce the modifier and Ado concentrations. Control experiments show that dilution did not re-activate the enzyme. The activity and fluorescence were determined before and after DTT treatment. Corrections for inner filter effect were made using the formula described in the Experimental section. After excitation at 295 nm, emission was measured at 340 nm.

Experiment	Remaining fluorescence (%)	Remaining activity (%)
(A) Enz + arsenite–BAL	55	3
Enz + Ado + arsenite–BAL	95	90
Enz + arsenite–BAL + DTT	90	95
(B) Enz + MMTS	87	5
Enz + Ado + MMTS	94	89
Enz + MMTS + DTT	92	87

Figure 6 Stern–Volmer plot of fluorescence quenching by KI (A) and acrylamide (B) of native (○), Ado-protected (△) and dithiol-modified (●) enzyme

The wavelengths for excitation and emission were 295 and 340 nm respectively. Refer to text for details.

substrate complex supports the commonality of the protective and catalytic sites.

Double-inhibitor experiment

The ability to protect the enzyme by Ado against inactivation by either arsenite–BAL or other monothiol blockers including NEM and MMTS [25] provided us with a method of verifying whether inactivation was due to exclusive trapping of the same two thiol residues located at the active site. To test this point, a two-stage modification procedure was performed. NEM, because of its obvious advantages, was used as the first modifier along with diamide or arsenite–BAL as the subsequent blocker. In the first step, the Ado (800 μ M)-protected enzyme was incubated for 20 min with NEM (400 μ M) in order to block other accessible non-essential thiols. The excess reactants were separated from the enzyme by extensive dialysis. The dialysed enzyme, on treatment with arsenite–BAL and diamide, displayed inhibitory

kinetics similar to those of enzyme that had not undergone prior NEM treatment (compare Figure 4 and Figure 1). As expected, exposure of this inactivated enzyme to DTT led to restoration of activity. Since NEM modification is irreversible, the result suggested exclusive involvement of the same two vicinal thiols which were protected by Ado during initial NEM treatment.

Substrate-binding properties of the modified enzymes

Intermolecular interaction between substrate and enzyme forms the basis of enzyme catalysis [33]. In order to get a clearer picture of the binding behaviour, the specific affinity of Adk for the 5'-AMP–Sephacrose column was utilized. Inasmuch as 5'-AMP is a competitive inhibitor of Ado phosphorylation [7], the lack of binding of the enzyme to 5'-AMP–Sephacrose 4B can be taken as loss of Ado-binding ability. The results demonstrated that, unlike the native enzyme, most of the arsenite–BAL-inactivated enzyme (80 % inactive) failed to bind to the column (Figure 5B). From this experiment it may be argued that inactivation resulted in non-specific modification and hence loss of binding ability. However, such a possibility was ruled out when it was observed that Ado-protected enzyme behaved like the unmodified enzyme (compare Figures 5C and 5A). Moreover, a completely inactivated enzyme fraction, after DTT treatment, also behaved like the unmodified enzyme (Figure 5D). Western-blot analysis of the flow-through and Ado-eluted fractions indicated that the respective amounts of protein distributed in the two fractions correlated well with the extent of modification as determined by the activity-protection assay. The loss of binding ability observed could be due to either modification of the target site for binding or inaccessibility of the binding site. In order to distinguish between the two possibilities, the enzyme was treated with MMTS to complete inactivation. Interestingly, most of the MMTS-modified enzyme did bind to the column and was eluted with Ado, indicating non-participation of the -SH groups in the binding processes (data not shown).

Fluorescence studies

Ado-induced quenching of enzyme fluorescence was observed previously [17,34]. The present results indicate that exposure of the enzyme to arsenite–BAL partially impaired protein fluorescence with concomitant loss of activity. DTT regenerated both protein fluorescence and catalytic activity (Table 2). In contrast, the loss of activity observed after MMTS modification was not accompanied by significant fluorescence loss. Since MMTS modification does not cause major conformational alteration, the results suggest that the conformational tightening of the active site due to formation of an -S-S- bridge was responsible for the loss of fluorescence in arsenite–BAL-treated enzyme [35]. Clearly, the primary cause of enzyme inactivation was oxidation or alkylation of the -SH groups.

Environment of the active site

In order to analyse the effects of modification on fluorophore exposure and to study the active-site environment of the enzyme, the classical Stern–Volmer law along with its modified version (as proposed by Lehrer) were applied to native, Ado-complexed and arsenite–BAL-modified enzymes.

Studies with native enzyme

The Stern–Volmer plot of fluorescence quenching of native enzyme, when exposed to iodide and acrylamide, showed downward curvature indicating the existence of only collisional quenching [36,37] (Figure 6). The quenching parameters f_a and

Table 3 Fluorescence quenching parameters from modified Stern–Volmer (Lehrer) plots

The concentration of Adk was kept at 0.8 μM . Saturating amounts of Ado (100 μM) were added in order to form enzyme–substrate complex. After modification of native and Ado-protected (1 mM) enzyme with 150 μM arsenite–BAL, the samples were dialysed extensively to remove free Ado and arsenite–BAL. Denaturation of the enzyme was carried out by overnight incubation in the presence of 6 M guanidinium chloride and 30 mM DTT. Excitation and emission were at 295 and 340 nm respectively.

Quencher	Enzyme	[Q] (mM)	K_{sv} (M^{-1})	f_a
Acrylamide	Denatured	0–500	18.0	1.0
	Native	0–500	9.5	1.0
	Ado-complexed	0–500	6.6	1.0
	Modified	0–500	7.0	1.0
	Ado-protected	0–500	9.0	1.0
KI	Denatured	0–500	15.0	1.0
	Native	0–75	11.0	0.30
		75–500	6.6	0.71
	Ado-complexed	0–75	10.0	0.25
		75–500	5.7	0.55
	Modified	0–500	6.0	1.0
	Ado-protected	0–75	11.0	0.30
		75–500	6.35	0.71

K_{sv} , obtained from linear regions of the modified Stern–Volmer plot, indicated that only 30% of the emission was accessible to low concentrations of iodide with a high apparent K_{sv} . Clearly, this is due to tryptophan residues located at the protein surface.

With increasing concentrations of iodide, a further 41% was accessible with comparatively lower K_{sv} (Table 3). In contrast, neutral polar quencher (acrylamide) was 100% accessible. Therefore the lower f_a value for iodide compared with acrylamide would be consistent with a relatively hydrophobic environment of these residues. Since 29% of the emission was resistant to iodide quenching, it is suggested that at least one tryptophan residue remained buried in the hydrophobic core of the protein [21].

Studies with Ado-complexed enzyme

The fluorescence quenching pattern (Stern–Volmer plot) of the Ado-complexed enzyme with both iodide and acrylamide produced a similar downward curvature to that of the native enzyme with the difference that iodide appeared to be more effective than acrylamide in detecting changes in active-site confirmation. The f_a for the Ado-complexed enzyme with iodide was 55% compared with 71% for native enzyme with a lower apparent K_{sv} . These results support the conclusion that besides the buried tryptophan, another tryptophan must be at the active site which is protected by Ado from interacting with iodide. In contrast, the entry of acrylamide, because of its neutral polar nature, was not hindered.

Studies with chemically modified enzyme

On exposure to arsenite–BAL, the emission of the enzyme was red-shifted by 3 nm. Addition of increasing concentrations of iodide and acrylamide resulted in a further red shift (results not shown). The red shift of 3 nm induced on modification is indicative of the fact that the contribution of the tryptophan(s),

Figure 7 Schematic model showing structure of the active site of Adk and conformational alteration that occurs as the result of modification by dithiol and monothiol modifiers

Relative positions of the amino acids within the active site are empirical.

the emission of which is in the blue part of the spectrum, is missing. Interestingly, the f_a value of the modified enzyme for both iodide and acrylamide was found to be 1 (Table 3). In contrast, the Ado-protected enzyme, after modification, yielded K_{sv} and f_a values similar to those of the native enzyme. Taken together, these results provide strong support for the interpretation that the hydrophobic active site of *L. donovani* Adk contains two conformationally vicinal thiols and at least one tryptophan residue.

DISCUSSION

In contrast with Adk from hamster liver, exposure of *L. donovani* Adk to arsenite-BAL and diamide led to dramatic loss of activity, consistent with the formation of a cystine bridge. From linear correlation of the extent of disulphide bridge formation with loss of activity and substrate-protection studies, it was concluded that inactivation was caused by modification of one pair of spatially vicinal thiols located at the active site.

In one of our previous studies, it was demonstrated that *L. donovani* Adk displays a characteristic tryptophan fluorescence which was reduced by addition of Ado [17]. The present study demonstrates that, of the four tryptophan residues present in the protein, at least one is located at the active site. Since arsenite-BAL treatment led to loss of fluorescence, these parameters provided us with the unique opportunity to probe the enzyme's active-site environment and study the change in its overall conformation. Fluorescence-quenching studies, activity assays and substrate-binding experiments led us to make the following inferences: (i) the loss of conformational flexibility imposed by formation of an -S-S- bridge was responsible for the impaired substrate binding resulting in catalytic inactivation and (ii) the inactivation of the enzyme by MMTS, without the concomitant change in fluorescence pattern and substrate-binding ability, resulted from thiol group oxidation or alkylation. Clearly the -SH group(s) of the enzyme must have some important function during catalysis.

We also utilized small-molecule fluorescence quenchers to detect differences in the accessible emission of unmodified and dithiol-modified enzymes. As expected for a multitryptophan protein, exposure to various quenchers led to heterogeneous emission spectra (data not presented). The fraction of accessible emission (f_a) for native enzyme was lower for iodide and acrylamide, suggesting that the tryptophan environment is highly hydrophobic. Exposure of the Ado-complexed enzyme to iodide led to further lowering of both f_a and K_{sv} , indicating conformational tightening of the active site. In contrast, the quenching parameters (K_{sv} and f_a) of dithiol-modified enzyme show 100% accessibility for both iodide and acrylamide, indicating a change in ionic environment. As expected, Ado-protected enzyme, despite arsenite-BAL treatment, behaved like native enzyme.

It was previously demonstrated that a single arginine residue, located at the active site, was responsible for binding of Ado to the enzyme [17]. From the present studies it is evident that at least one tryptophan and two conformationally vicinal thiols also form a part of the hydrophobic Ado-binding domain. On the basis of these results, a model of the Ado-binding site is proposed (Figure 7). The model, as shown, is consistent with all the experimental results described herein and should serve as a first step towards further investigation of the active-site structure of *L. donovani* Adk.

A role for vicinally located thiol pairs that are presumed to be

involved in the catalytic function of a number of enzymes including pyruvate kinase [38], phosphoenolpyruvate carboxykinase [39] and mevalonate 5'-diphosphate carboxylase [40] has been described. It has been suggested that vicinal thiols may be a general feature of enzymes having phosphate-binding sites [41]. Our present finding of vicinal thiols in *L. donovani* but not in hamster liver Adk, casts a doubt on such a generalization.

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