Reversal of ADP-Mediated Aggregation of Adenosine Kinase by Cyclophilin Leads to Its Reactivation†

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ABSTRACT: Cyclophilins have been implicated in several important cellular functions. Our earlier results showed that reactivation of adenosine kinase (AdK) by CyP (LdCyP) from the parasitic protozoa *Leishmania donovani* is accompanied with disaggregation of the enzyme [Chakraborty, A., et al. (2002) *J. Biol. Chem. 277*, 47451−47460; Chakraborty, A., et al. (2004) *Biochemistry 43*, 11862−11872]. However, it remained to be known why the enzyme displayed progressive inhibition during the time-dependent reaction and what LdCyP does to prevent and/or reverse the inhibition. Herein, we demonstrate that one of its reaction products, ADP but not AMP, facilitates the formation of AdK aggregates, leading to its inactivation. Further studies revealed that LdCyP reactivates the enzyme by withdrawing the ADP inhibition. To investigate the molecular mechanism, the intrinsic tryptophan fluorescence and polarization of AdK were monitored in the presence of either LdCyP or ADP and in combination thereof. Whereas in the presence of LdCyP the tryptophan fluorescence emission maxima of AdK exhibited a red shift, ADP had a quenching effect. However, both the red shift and quenching became less noticeable when one (W234) of the two tryptophan residues of AdK was altered, indicating W234 fluorescence is relatively more sensitive to both LdCyP and ADP binding. Kinetic measurements indicated that LdCyP-facilitated reactivation of AdK is accompanied with a concomitant increase in the K_D of ADP but not of AMP. Interestingly, addition of myokinase (MK) and pyruvate kinase (PK) along with phosphoenolpyruvate, either singly or in conjunction, to the AdK reaction mixture led to its reactivation. The effect of PK but not of MK could be substituted by CyP and vice versa. Taken together, the results suggest that LdCyP-induced reactivation occurs due to conformational reorientation of AdK in a manner that decreases the affinity of the enzyme for ADP with consequent relief from the ADP-mediated aggregation.

Cyclophilins (CyPs),[1] a multigenic family of ubiquitous proteins carrying peptidyl-prolyl *cis*−*trans* isomerase (PPIase) activity, are the specific receptors of the immunosuppressive drug cyclosporin A (CsA) (*1*, *2*). Besides mediating the CsA-induced immunosuppression, CyPs are involved in various cellular processes that span from cell division, receptor maturation, acceleration of protein folding, and protection against human immunodeficiency virus (HIV) infection to several other processes (*3*−*13*). Alterations of activities of various enzymes and transcription factors, following interaction with CyP, have also been reported (*14*−*16*). In another report, it has been shown that CyP keeps peroxiredoxin proteins activated by keeping the enzyme in the reduced state (*17*).

Leishmania donovani, a dimorphic purine auxotrophic parasitic protozoon, is the causative agent for human kala-azar. This parasite exists as a flagellated promastigote (extracellular form) in the sand fly vector and is transformed into amastigote (intracellular form) in the mammalian macrophages. During the process of transformation, the activity of a large number of proteins has been reported to be stage-specifically altered (*18*, *19*).

Adenosine kinase (AdK), one of the purine salvage pathway enzymes, which by following a sequential bi-bi orderly mechanism carries out phosphorylation of Ado in the presence ATP to form AMP and ADP, plays a pivotal role in the metabolism and uptake of Ado, a key modulator of various cellular functions (*20*, *21*). Moreover, the fact that the activity of this enzyme from all known sources is regulated by its substrates and reaction products has been known for a long time (*22*−*25*). In *L. donovani*, the enzyme has been shown to display several-fold higher activity upon transformation into amastigotes (*26*). However, to date, no evidence exists to indicate that the higher activity observed was due to an acceleration of protein synthesis.

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[1] Abbreviations: DTT, dithiothreitol; PK, pyruvate kinase; MK, myokinase; AdK, adenosine kinase; CsA, cyclosporin A; LdCyP, *Leishmania donovani* cyclophilin; CyP, cyclophilin; Ado, adenosine; PEP, phosphoenolpyruvate; FKBP, FK506 binding protein; Ni−NTA, nickel−nitrilotriacetic acid.

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Recently, we demonstrated that AdK, which has an inherent tendency to form inactive soluble aggregates, could be disaggregated by a CyP (LdCyP) in an isomerase-independent fashion, resulting in reactivation. The reactivation of AdK was achieved in vitro with a stoichiometric amount of LdCyP and in cells coexpressing both of the proteins, thus indicating interaction is relatively specific (*27*, *28*). However, the results of these parallel experiments failed to provide any direct evidence correlating LdCyP-mediated disaggregation of the enzyme with its reactivation. Moreover, the question as to why the rate of the enzymatic reaction decreased progressively with time until it showed almost complete inhibition could not be answered from those experiments. On the other hand, two recent independently published reports claimed that both CsA and FK506, another immunosuppressive drug, inhibit Ado uptake in T-lymphocyte and endothelial cells, respectively, by a mechanism that involves inhibition of AdK activity (*29*, *30*). Since the majority of the effects of these immunosuppressants are known to be mediated through the formation of a complex between these drugs and their corresponding specific immunophilins, CyP and FKBP, respectively, it obviously becomes important to know whether the reactivation of AdK by LdCyP has any relationship with such a process.

While trying to comprehend the missing link, we felt that detailed knowledge of the molecular mechanism by which LdCyP reactivates AdK may provide some important clues toward understanding the process. In this communication, we demonstrate that AdK, in addition to its inherent tendency to aggregate by itself, forms large oligomers during the reaction due to the generation of ADP, one of its reaction products. LdCyP, while causing disaggregation of these oligomers, induces subtle structural remodeling of the enzyme around its active site in a manner that reduced the apparent affinity of AdK for ADP, resulting in ultimate relief of ADP-mediated product inhibition and subsequent enhancement of substrate turnover. The mechanism of CyP-mediated stimulation of AdK described here is novel and deviates from the hitherto known roles of CyP, thus raising the possibility of a regulatory mechanism mediated by the interplay of CyP and one of the reaction products, ADP, of the enzymatic reaction.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all chemicals were of analytical grade and were obtained from Sigma Chemicals.

Site-Specific Mutagenesis. Wild-type LdCyP harbors only a single "Trp" residue at position 122 that overlaps with the CsA binding motif (WLDGRHVVFG) (*31*). Since the LdCyP-mediated chaperone function described here is CsA-independent, we mutated residue W122 to G using Stratagene's QuickChange kit. The mutant LdCyP (mLdCyP), despite losing its intrinsic "Trp" fluorescence property, was equally efficient in reactivating AdK (Figure 1). To carry out mutation, the N-terminal His_6-tagged expression plasmid (pQE32) harboring the wild-type LdCyP gene was used as the template. The following sense primer along with the antisense counterpart, with its substitution site underlined, was used: LdCyP (W122G), 5′-GCGCCGACGCCG**GGG**-CTTGATGGCCGCC-3′. The same procedure using pQE30 harboring the wild-type AdK gene was adapted for mutation of the W73 and W234 residues of AdK to arginine.

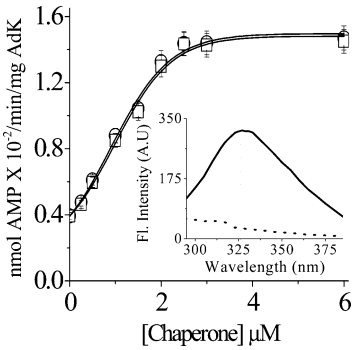

FIGURE 1: Effect of the wild type (○) and mLdCyP (□) on the reactivation pattern of AdK. The inset shows the intrinsic tryptophan fluorescence emission spectra of the wild type (—) and mLdCyP (•••). Excitation was performed at 286 nm. Cyclophilin concentration was maintained at 2 μM.

The following sense primers, along with their antisense counterparts, were used. The substitution sites are underlined: AdK (W73R), 5′-CGCGTGGCGCAG**CGG**ATCGCG-CAGGCC-3′; and AdK (W234R), 5′-GCGGACGCCAT-GAAG**CGG**AACCCCGCCAGCACC-3′. Mutations were confirmed by automated DNA sequencing in a Perkin-Elmer ABI Prism DNA sequencer.

Expression and Purification of Wild-Type and Mutant LdCyP and AdK. The mLdCyP plasmid DNA generated was used as such for expression. *Escherichia coli* M15 [pREP4] cell clones harboring the wild-type and mutant plasmids were used for IPTG (0.5 mM) induction. Following induction for 3 h at 26 °C, both the wild-type and mutant proteins were purified to homogeneity using a Ni−NTA−agarose column as per the suggested procedure of the manufacturer. In brief, the induced cells were harvested and subjected to lysis using buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mg/mL lysozyme. The resultant suspension was sonicated and centrifuged at 27000*g* for 30 min at 4 °C. The supernatant was directly loaded onto a pre-equilibrated Ni−NTA column. Then the column was washed with the buffer containing 20 mM imidazole. The bound proteins were then eluted with elution buffer containing 250 mM imidazole. For all the biochemical experiments, imidazole-eluted His_6-tagged cyclophilins were extensively dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5) and 1% glycerol. The purified homogeneous proteins were free from detectable pyruvate kinase (PK) and myokinase (MK) activities. The methods of expression and purification of the wild type and different AdK mutants were same except that 1 mM dithiothreitol was used in the dialysis buffer (*32*).

AdK Reactivation Assay. The reactivation of AdK was monitored under saturating substrate concentrations using the radiochemical assay procedure described previously (*27*). Wherever indicated, LdCyP, MK, PK, and phosphoenolpyruvate (PEP) were added at final concentrations of 5 μM, 27 IU/mL, 18 IU/mL, and 1 mM, respectively (*33*). For product inhibition studies with ADP and AMP, assays were performed for 15 min at 30 °C in the presence of 400 μM ATP. Different concentrations of inhibitors were used as indicated.

Fluorescence, Polarization, and Light Scattering Measurements. The intrinsic tryptophan fluorescence of AdK was monitored using a Hitachi F-4500 spectrofluorimeter as described in the previous report (*28*). To study the pattern

of interaction of AdK with LdCyP, the nonfluorescent mLdCyP, wherever indicated, was used at increasing concentrations. The buffer contained 20 mM Tris-HCl (pH 7.5) and 1 mM DTT. Magnesium chloride (1 mM) was included for ligand binding experiments. The concentration of AdK was maintained at 1 μM. The binding of nucleoside and nucleotides to the enzyme was monitored by ligand-induced fluorescence quenching with the assumption that fluorescence change was directly proportional to the concentration of the enzyme−ligand complex, and the molar fraction (υ) of the enzyme bound at each concentration of the ligand was given by (*34*, *35*)

$$\upsilon = [\mathrm{EL}]/[\mathrm{E}]_{\mathrm{total}} = \Delta F/\Delta F_{\mathrm{max}}$$

where the enzyme concentration is expressed in normality (i.e., active site concentration), ΔF is the actual fluorescence change at a given concentration of the ligand, and ΔF_{max} is the maximum fluorescence change at the saturating concentration of the ligand. The correct evaluation of ΔF_{max} was obtained from a double-reciprocal plot of ΔF versus ligand concentration. K_{D} was calculated from the Scatchard analysis.

Quenching experiments were carried out using acrylamide and KI. To maintain a constant ionic strength and to prevent I_3^- formation, a 5 M solution of KI was made in 1 M KCl containing trace amounts of sodium thiosulfate (*36*). Acrylamide (8 M) was made up in water. The activity of the enzyme remained unaffected even at the highest concentrations of quencher used; however, the cyclophilin-mediated stimulation of AdK started to show slight inhibition at concentrations of KI beyond 0.5 M. On the basis of the nature of the plot of F_o/F versus Q, it appeared that the mode of quenching was mostly collisional with heterogeneity (*37*), thus reducing the original Stern−Volmer equation to $F_o/F = 1 + K_{sv}[\mathrm{Q}]$, where F_o and F are the fluorescence in the absence and presence of quenchers, respectively, [Q] is the concentration of the quenchers, and K_{sv} is the Stern−Volmer constant. The concentrations of AdK and mLdCyP were maintained at 1 and 2 μM, respectively.

Fluorescence polarization measurement of AdK (1 μM) was carried out at 25 °C in the presence of increasing concentrations of mLdCyP, ADP, and in combination thereof (as indicated in figure). Excitation and emission were at 295 and 333 nm, respectively. Slit widths were 5 nm. The polarization was calculated by using the formula $P = (I_{||} - GI_{\perp})/(I_{||} + GI_{\perp})$, where P represents the polarization value, $I_{||}$ the fluorescence intensity at the parallel position of the polarizer, and $I_{\perp}$ the fluorescence intensity at the perpendicular position of the polarizer. The G-factor was calculated to be 0.9095 (*37*).

Light scattering measurements of AdK (3 μM) were carried out at 25 °C in the presence of different concentrations of nucleosides and nucleotides. The excitation and emission wavelengths were fixed at 350 nm, where AdK and LdCyP had no absorption. Slit widths were 5 nm. Ado, AMP, ADP, and ATP were used at increasing concentrations (as indicated in the figure legends). The buffer used was similar to the one used in the fluorometric experiments (*27*).

RESULTS

AdK Undergoes Aggregation during the Reaction. Increased inactivation of the enzyme in the absence of LdCyP led us to assess its state of oligomerization during the kinetic reaction. Results show that in the absence of LdCyP, the extent of aggregation, as observed from the accumulation of the protein at the top of the gel, gradually increased with the progress of the reaction, concomitant with the decrease in the concentration of the protein in the lower molecular size ranges (Figure 2A, lanes 3−6). However, when the reaction was allowed to proceed in the presence of LdCyP, the appearance of the high-molecular weight band, as measured by the same parameter, was much more delayed as compared to that in the reaction without LdCyP, indicating formation of reduced amounts of aggregates (Figure 2A, lanes 7−10). Interestingly, when LdCyP was absent from the reaction mixture, the multiple oligomers seen before the initiation of the reaction (Figure 2A, lanes 1−3) almost vanished with time, suggesting a clear possibility of conversion of these multimeric oligomers into much larger aggregates (Figure 2A, lanes 4−6). Parallel activity analysis of these samples at the corresponding times also showed a trend analogous with the aggregation profile, indicating a direct relationship between aggregation and inactivation (Figure 2B). Clearly, the results are consistent with the interpretation that LdCyP indeed prevents aggregation of the enzyme during the reaction.

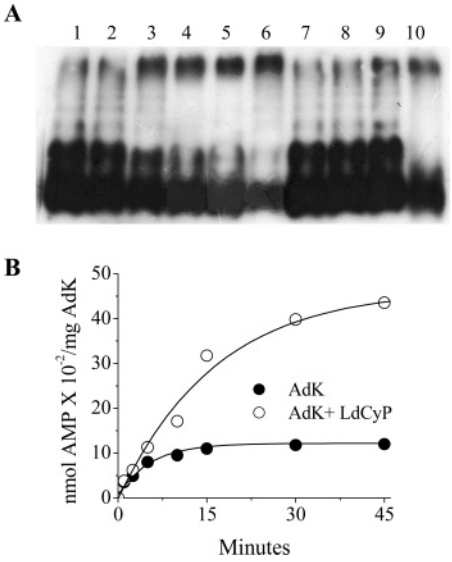

FIGURE 2: Aggregation of AdK occurs during the reaction. Two complete sets of AdK (100 nM) reactions, with or without LdCyP (5 μM), were allowed to proceed in parallel. At the indicated times, four equal aliquots from both of the sets (two from each) were withdrawn and mixed with an appropriate amount of EDTA to stop the reaction. After 30 min, samples from each sets were run on 10% native PAGE and analyzed by immunoblotting with AdK polyclonal antibodies (A), while the replica samples from the same sets were analyzed for enzymatic activity in the presence (○) and absence (●) of LdCyP, following the regular procedure (B). Lane 1 shows oligomeric status of the same amount of AdK kept at 30 °C for 30 min without any reaction. Lanes 2−6 show the oligomeric status of AdK at 0, 2.5, 5, 10, and 30 min, respectively, assayed without LdCyP, whereas lanes 7−10 represent 0, 2.5, 5, and 30 min samples, respectively, assayed with LdCyP.

ADP Facilitates AdK Aggregation. Product-mediated aggregation and disaggregation is one of the mechanisms by which activities of many proteins and/or enzymes are regulated (*38*, *39*). Moreover, studies from various laboratories have shown that substrates and reaction products have important regulatory roles in various enzymatic reactions

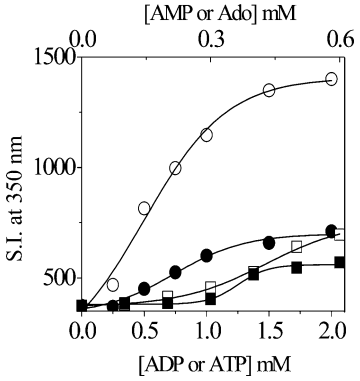

FIGURE 3: ADP facilitates AdK aggregation. The scattering intensity of AdK (3 μM) was measured in the presence of increasing concentrations of Ado (■), AMP (□), ADP (○), and ATP (●). For details, see Materials and Methods.

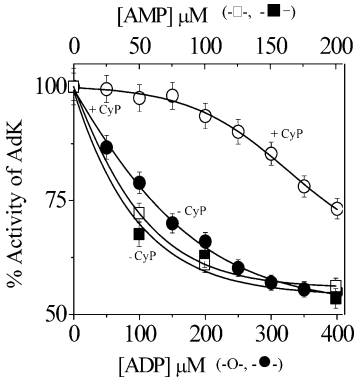

FIGURE 4: CyP prevents ADP but not AMP-mediated AdK inhibition. The inhibition of AdK (50 nM) activity by ADP (○ and ●) and AMP (□ and ■) was monitored in the presence (○ and □) and absence (● and ■) of 5 μM CyP.

(*40*−*42*). Since AdKs from all sources are known to be inhibited by both its reaction products (*22*−*25*), our earlier work demonstrating slow inactivation of AdK during time-dependent reactions and increased oligomerization under reaction conditions provoked us to investigate whether the substrates or products of AdK reaction had any effect on the enzyme aggregation and subsequent inhibition. To investigate this possibility, the scattering intensity of AdK was monitored in the presence of increasing concentrations of substrates and its products (Figure 3). Interestingly, addition of increasing amounts of one of its product ADP to a fixed amount of the protein led to an appreciable increase in the scattering intensity of the AdK, suggesting ADP-facilitated aggregation of the protein. In comparison, Ado, AMP, and ATP had a much weaker effect (≤40%) on the scattering of the protein.

Withdrawal of ADP-Mediated Inhibition of AdK by LdCyP. Having confirmed that ADP-facilitated aggregation of the protein resulted in its inhibition, we pondered the possibility of whether LdCyP had any effect on the product-mediated inhibition of AdK (*22*). Indeed, the results of the inhibition studies carried out in the presence of increasing concentrations of exogenous ADP and AMP indicated that LdCyP clearly reversed the inhibitory effect of ADP (Figure 4). In contrast, LdCyP had practically no effect on the AMP-mediated inhibition. From these results, it is concluded that LdCyP, by reversing the ADP-mediated aggregation, probably keeps the enzyme active. The fact that AMP, despite

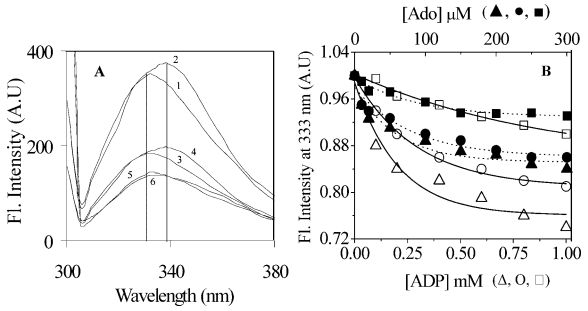

FIGURE 5: mLdCyP-mediated modulation of tryptophan fluorescence emission maxima of AdK. (A) The fluorescence emission spectra of AdK (1 μM, spectrum 1) were monitored in the presence of mLdCyP (2 μM, spectrum 2). Curves 3 and 4 show the emission spectra of W73R mutant AdK measured in the absence and presence of mLdCyP, respectively, whereas curves 5 and 6 display fluorescent emission spectra of the W234R AdK mutant in the absence and presence of the chaperone. The concentrations of both the mutant AdK proteins were maintained at 1 μM in all the experiments. (B) Quenching of the tryptophan fluorescence intensity of wild-type (△ and ▲), W73R (○ and ●), and W234R (□ and ■) AdK proteins at 333 nm monitored as a function of ADP and Ado concentrations, respectively, as indicated. The initial fluorescence intensity in arbitrary units (AU) of wild-type and mutant AdK without any ligands was considered to be unity. The concentrations of wild-type and mutant AdK proteins were maintained at 1 μM.

being an inhibitor, did neither aggregate AdK appreciably (Figure 2) or could be competed out by LdCyP in the inhibition reaction is consistent with the interpretation that the mode of inhibition by two of its products follows different mechanisms, a conclusion that will be further validated in the subsequent sections.

Interaction Sites of LdCyP and ADP on AdK Are Conformationally Vicinal. Like AdKs from most sources, the intrinsic tryptophan fluorescence of *L. donovani* AdK, harboring two tryptophan residues (W73 and W234), is sensitive to both substrate and product binding (*43*−*45*). Moreover, results from our preceding section suggest that ADP inhibition of AdK can be withdrawn by LdCyP. In view of the mitigating effect of LdCyP on the ADP inhibition of the enzyme, we were interested in investigating whether the reactivation process can be understood on the basis of the structural properties of the enzyme (*46*, *47*). Results show that upon addition of the tryptophan fluorescence negative mLdCyP, the tryptophan emission maxima of AdK (Figure 5A, curve 1) red shifted from 333 to 339 nm (Figure 5A, curve 2). Such a large red shift (6 nm) in the emission maxima, attributable to the change in the polarity of the microenvironment around the Trp residues due to their exposure to the solvent face (*48*, *49*), could result from either (i) conformational modulation, (ii) disaggregation, or (iii) a combination of both. Regardless of the cause, this observation, nevertheless, allowed us to design a strategy for probing the binding sites of LdCyP and ADP on the enzyme. To have such information, we generated two mutants (W73R and W234R) of AdK. Interestingly, upon addition of mLdCyP, the W73R mutant protein produced a red shift similar to that of the wild-type protein (Figure 5A, curves 3 and 4). In contrast, LdCyP exerted little effect on the fluorescence of the W234R mutant protein (Figure 5A, curves 5 and 6). In parallel experiments that followed the quenching pattern of AdK fluorescence as a function of ADP concentration, it also became evident that the extent of fluorescence quenching

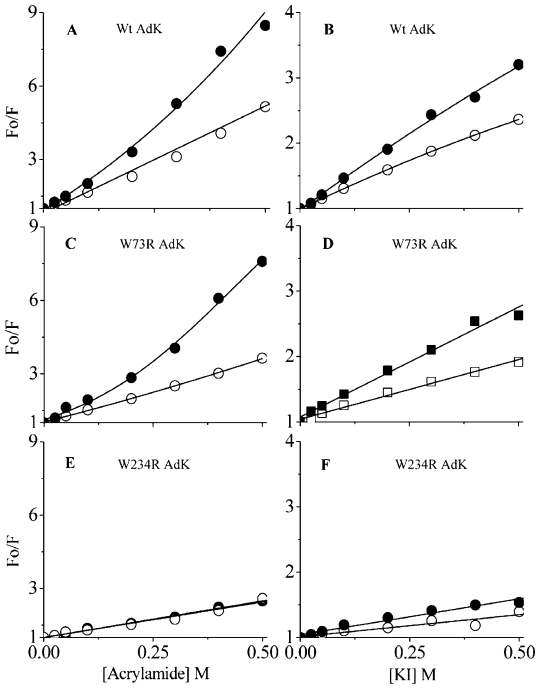

FIGURE 6: Stern−Volmer analyses of fluorescence quenching by acrylamide (A, C, and E) and KI (B, D, and F) of wild-type (A and B), W73R (C and D), and W234R (E and F) AdK enzymes were carried out in the presence (●) and absence (○) of LdCyP.

of the W73R mutant was much higher than that of the W234R mutant in response to ADP binding (Figure 5B). Moreover, the quantum of quenching for both the mutants was more or less similar with both ATP and ADP. In contrast, the quenching with Ado and AMP was much less (data not shown). Therefore, the LdCyP-induced red shift of the emission peak as opposed to quenching of the enzyme fluorescence by substrates and products is indicative of the fact that their modes of recognition of the enzyme are different. Moreover, these results are consistent with the interpretation that the sites where LdCyP and ADP interact with the AdK are most probably conformationally vicinal and lie proximal to the W234 residue.

Environment of the Two Tryptophan Residues. The differential effects of LdCyP on the fluorescence emission property of the two tryptophan mutants led us to investigate the environment around these residues. To ascertain this, the effect of LdCyP on the fluorescence quenching characteristics of the wild type and its two mutant proteins (W73R and W234R) was monitored in the presence of increasing concentrations of acrylamide and KI (Figure 6). In the presence of LdCyP, the Stern−Volmer plots of both the native and W73R mutant proteins displayed characteristics signifying increased accessibility of the fluorophore (Figure 6A−D). In contrast, LdCyP exerted almost no effect on the quenching pattern of the W234R mutant (Figure 6E,F). Furthermore, the mixed quenching as observed in the presence of acrylamide (Figure 6A,C) as opposed to the static quenching in the presence of KI (Figure 6B,D), apart from supporting the conclusion of the preceding section, appears to suggest that the W73 residue is comparatively less solvent exposed than the W234 residue (*37*). CsCl had no effect on any of the proteins.

LdCyP Reduces the Affinity of AdK for ADP. Aggregation−disaggregation of macromolecules, which is often

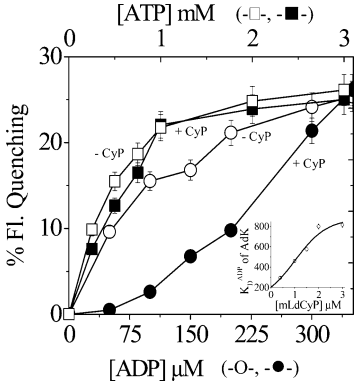

FIGURE 7: Effect of mLdCyP on the ligand-induced fluorescence quenching pattern of wild-type AdK. The intrinsic Trp fluorescence quenching of AdK (1 μM) in the presence (■ and ●) or absence (○ and □) of mLdCyP (2 μM) was followed as a function of ADP (● and ○) and ATP (■ and □) concentrations. The fluorescence intensity of AdK without any addition taken as 100% was plotted at the origin of the curve. Inset, the K_D of ADP toward AdK was plotted against increasing concentrations of mLdCyP. Calculations, as described in Materials and Methods, were carried out accordingly.

Table 1: Dissociation Constants (K_D) of Substrates and Products of AdK in the Presence and Absence of mLdCyP[a]

ligand	K_D LdAdK (μM) (without LdCyP)	K_D LdAdK (μM) (with LdCyP)
Ado	21 ± 2	35 ± 2.5
AMP	98 ± 4	92 ± 4
ADP	200 ± 8	850 ± 15
ATP	325 ± 8	315 ± 8

[a] K_D was determined using the procedure described in Materials and Methods. The concentrations of AdK and mLdCyP were maintained at 1 and 2 μM, respectively.

associated with conformational alteration, may alter the binding properties of the ligands (*46*, *50*). To investigate whether the interaction of CyP with AdK exerted any influence on AdK in terms of its substrate and product binding properties, the dissociation constants (K_D) of all the substrates and products, in absence and presence of the chaperone, were determined. Since the intrinsic Trp fluorescence of AdK from all sources is known to be quenched by all its substrates and products, this parameter was used to follow the effect of the mLdCyP on the binding of substrates and products to AdK (*43*, *44*). In Figure 7, the percent fluorescence quenching of AdK (1 μM) as a function of ADP and ATP concentrations are displayed both in the absence and in the presence of mLdCyP (2 μM). It is evident that while the chaperone exerted little influence on the ATP-mediated fluorescence quenching of AdK, it had a profound effect on the ADP-mediated fluorescence quenching pattern. In the presence of a fixed amount of chaperone, ADP, at lower concentrations, clearly failed to exert the same quenching effect and showed a lag as compared to the reaction set without the chaperone. The fluorescence quenching pattern in the presence of Ado and AMP also did not show any significant differences upon addition of the chaperone (data not shown). From Figure 7 (inset) and Table 1, it is evident that while the binding affinities of Ado, AMP, and ATP with or without mLdCyP remained more or less same, the K_D of ADP, in the presence of the mutant chaperone, increased from 200 ± 8 to 850 ± 15 μM. These observations are consistent with the interpretation that

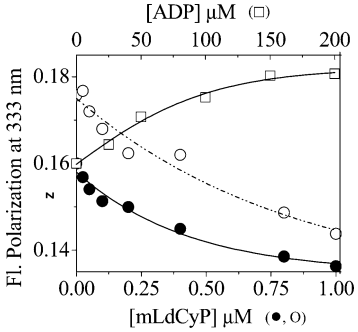

FIGURE 8: Effects of varying concentrations of mLdCyP (○ and ●) and ADP (□) on the oligomeric status of AdK as measured by fluorescence polarization. In the experiment representing the curve (-••-), the same amount of AdK, pretreated with 150 μM ADP, was used as the substrate for chaperone-induced disaggregation. The AdK concentration was fixed at 1 μM in all experimentals.

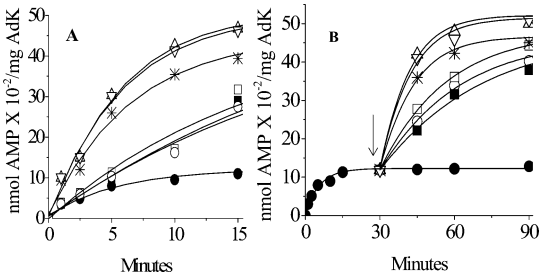

FIGURE 9: PK, MK, and CyP reactivate AdK. (A) The progress of Ado phosphorylation by AdK (50 nM) was followed in the presence of saturating amounts of PK, MK, CyP, and combinations thereof. For details, see Materials and Methods. (B) Addition of PK, MK, CyP, or a combination of these, as indicated, was made at the indicated time (↓). The notations are as follows: AdK without any addition (●), PK (■), MK (∗), CyP (○), PK with CyP (□), PK with MK (△), and MK with CyP (▽).

LdCyP-induced disaggregation of AdK must be associated with structural modulation of the enzyme in a manner that ultimately leads to a decrease in its affinity for ADP.

LdCyP Disperses ADP-Induced AdK Aggregates. Restricted rotational movement of macromolecules is directly proportional to its molecular volume and is monitored often by fluorescence polarization (*51*, *52*). This provided an opportunity to follow directly the effect of LdCyP on the AdK aggregates formed in the presence of ADP. As expected, the polarization value of the purified AdK increased gradually with the addition of increasing amounts of ADP, indicating that ADP facilitated aggregation (Figure 8). To investigate whether the chaperone was able to disaggregate the ADP-aggregated enzyme, increasing amounts of mLdCyP were added to the ADP-treated enzyme. Interestingly, LdCyP dispersed the ADP-aggregated enzyme in a fashion similar to that of the enzyme that was not treated with ADP. Of particular interest here is the observation that LdCyP, at concentrations above 0.5 μM, reduces the polarization values of ADP-treated AdK aggregates to a level below the polarization value of the same concentration of purified AdK without the chaperone. The results indicated that LdCyP disperses both forms of the enzyme aggregates, regardless of whether it was formed naturally or ADP-mediated.

LdCyP Function Can Be Mimicked by Pyruvate Kinase (PK). If accumulation of ADP is indeed the cause of AdK aggregation and its subsequent inactivation, it should be possible to reactivate the enzyme by depleting ADP from the reaction system. To have a direct proof in favor of this possibility, we modified the existing assay system in a manner that selectively minimized the accumulation of both of the products (Figure 9). Inclusion of saturating amounts of PK and MK separately or in combination in the reaction mixture served such a purpose. Using such an assay system, it was observed that both PK and MK alone or in conjunction stimulated the AdK activity to different extents (Figure 9A). While PK was ineffective without PEP, MK, by itself, was able to stimulate the activity, an indication of the importance of removal of both the products from the system. The higher rate of activation observed in the presence of MK over PK was most likely due to the lower inhibition constant (K_i) of AMP compared to that of ADP (*22*). Nevertheless, when they were added together, the rate of activation was nearly additive. Interestingly, in the absence of both PK and MK, the increased rate of AMP formation in the presence of LdCyP was almost identical to that found in the presence of PK. The rate did not show any tendency to increase even when PK was added along with LdCyP. However, the rate of stimulation increased significantly when PK was replaced with MK. Incidentally, the increased rate was similar to the rate attained in the presence of both PK and MK. The stimulatory effects of LdCyP, PK, and MK were the same regardless of the timing of the addition, indicating that the inhibition was indeed caused due to product accumulation (Figure 9B). Since CyP and PK did not have any cross contamination between themselves, the only likely explanation would be that both LdCyP and PK must be carrying out a function(s) that ultimately creates an environment favorable for AdK to carry on the forward reaction. It is known that PK, in the presence of PEP, can convert ADP to ATP (*53*) and as such has no effect on the aggregation status of AdK. However, like cyclophilin, PK could reduce the oligomerization of AdK during reaction conditions, indicating the involvement of ADP in the aggregation of the enzyme (unpublished observation). Taken together, these results, beyond any doubt, directly ratify all our earlier interpretations that CyP stimulates AdK activity by withdrawing ADP but not AMP inhibition.

DISCUSSION

The rapid, reversible, and noncatalytic mode of AdK reactivation upon addition of LdCyP led us to suspect that kinetic partitioning between the aggregated and disaggregated forms of the enzyme underlies the basis of the reactivation process (*27*). In our study, we show that (i) besides concentration-dependent inactivation, AdK also becomes inactivated during the reaction, (ii) LdCyP, by dispersing these ADP-induced aggregates, directly overcomes the ADP inhibition of the enzyme, (iii) the removal of ADP from the reaction mixture even by an alternate means leads to reactivation of the enzyme, and (iv) LdCyP increases the K_D of ADP for AdK with a concomitant increase in activity. From these observations, it appears that LdCyP most likely reactivates the enzyme by dispersing those oligomers that are formed in the presence of ADP, while the aggregates formed prior to the presence of ADP are probably stable and irreversible in nature and therefore remain inactive. This interpretation is consistent with our observations that (i) the rate of the reaction, even after preincubation of the enzyme

with LdCyP, hardly improves at the initial times of the reaction and the increase in the AMP yield is visible only after extended incubation under reaction conditions and (ii) the rate of production of AMP, after an initial burst for a few minutes, gradually tapers off with time. Characterization of these aggregates, which is going to be our future endeavor, is expected to shed further light in this regard.

The crystal structure of AdK from various sources revealed that, apart from some minor structural variations among themselves, the monomeric protein, in general, consists of two unequal-sized domains. The large domain is a three-layered sandwich of α-helices and β-sheets over which the small domain forms a lid. The substrates and products are known to be bound at the domain interfaces (*54*, *55*). Studies further showed that during the ordered sequential bi-bi reaction, the enzyme, following binding to the first substrate (Ado), undergoes a 30° hinge bending motion leading to domain closure. This allows ATP binding, followed by subsequent conformational modulation that results in the phospho transfer reaction and ultimate ordered release of the products (*22*, *23*, *56*). Because the substrates and the products occupy their respective specified sites, it is conceivable that the rate at which the products are released will have an important bearing on the overall rate of the forward reaction. In this context, of particular interest is our observation related to the differential effects of LdCyP on the withdrawal of AdK inhibition caused due to accumulation of both of the products. LdCyP-mediated reversal of ADP but not of AMP inhibition of the enzyme, coupled with the increase in the apparent dissociation constant (K_D) of ADP, suggested that LdCyP, following interaction with the enzyme, must be causing the conformational alteration of the enzyme. As a consequence, the enzyme becomes disaggregated, leading to the release of the ADP from the active site and resulting in an increased rate of the forward reaction. The fact that removal of ADP from the reaction mixture by PK also leads to reactivation of the enzyme supports such an interpretation. Inasmuch as AMP is known to inhibit the enzyme by competing with the Ado binding site and is the last product to be released from the enzyme surface, the failure to reverse AMP inhibition with LdCyP supports the interpretation that the chaperone most likely alters the local conformation close to the vicinity of the ATP and not the Ado binding site, resulting in an apparent increase in the K_D of ADP. Since, of the two Trp residues (W73 and W234) present in the *L. donovani* AdK, W234 fluorescence is relatively more sensitive to nucleotide (ATP or ADP) binding, the absence of a red shift in the emission maxima of the mutant AdK (W234R) but not of the W73R mutant in the presence of LdCyP is an additional experimental support in favor of such a conclusion. The effect of LdCyP on the fluorescence quenching of the enzyme, carried out in the presence of chemical quenchers, is also consistent with such a contention.

The fact that the process of nucleotide-induced aggregation−disaggregation of enzymes forms the basis of enzyme regulation has also been demonstrated with uridine kinase, thymidine kinase, and many other enzymes (*40*−*42*, *57*−*59*). Moreover, ligand or nucleotide induced structural alteration, which accounts for interaction of GroEL with its substrate or GroES and in the case of other proteins has been elegantly demonstrated (*60*−*63*). However, no report, to the best of our knowledge, has yet shown that CyP could

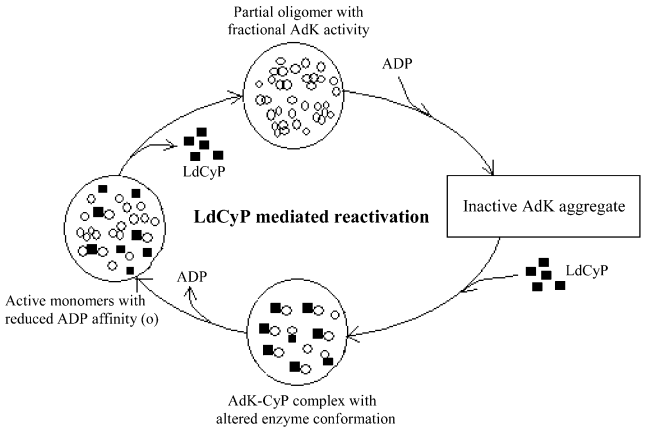

FIGURE 10: Schematic representation of the events of reactivation of AdK by LdCyP.

modulate the effects of nucleotides on nucleotide-binding enzymes. In this regard, it also needs to be mentioned that although both ADP and ATP appear to bind to the same pocket of the enzyme, LdCyP alters only ADP affinity and not ATP affinity, indicating subtle differences in the mode of binding of the two ligands. However, such differences in the mode of ATP and/or ADP binding, occurring due to either local conformational alteration of the nucleotide-binding motif or binding through different residues, are not unusual and have recently been noticed while studying the nucleotide binding properties of creatine kinase, apyrases, and Hsp70 proteins (*64*−*67*). Whether similar conformational modulation is responsible for discriminating the effect of LdCyP on binding of ADP or ATP to AdK, however, remains to be verified. Using these results as support, a composite schematic model depicting the sequence of events that probably leads to reactivation of AdK by LdCyP is presented in Figure 10. The postulated mechanism provides a logical explanation of how LdCyP reactivates the enzyme by dispersing the aggregates formed due to accumulation of ADP.

It is well-established that the intracellular adenine nucleotide concentrations, which form the largest nucleotide pool of most cells, are responsible for various important physiological functions (*21*). It is also well-known that AdK is the rate-limiting enzyme that regulates the concentrations of both extracellular Ado and the balance of intracellular adenine nucleotide pools necessary for ensuring ATP-mediated biological reactions (*21*, *68*). Since the K_i values of AMP and ADP for AdK inhibition are known to be in the range of their intracellular concentrations, the phenomenon of inhibition by its products is consistent with the concept of regulation by ATP charge (*69*). The concept calls for ATP conservation within the cell that is dependent on the [ATP]/[AMP] + [ADP] ratio so that the rate of reactions utilizing ATP remains low at decreased ATP concentrations. Given this scenario, the LdCyP-induced reactivation of AdK observed both under in vitro reaction conditions (*27*) and under simulated crowded in vivo conditions (*28*) may have important implications from the standpoint of enzyme regulation in vivo. A similar observation demonstrating functional solubilization of aggregation-prone HIV envelope proteins using SlyD or FkpA chaperones has very recently been reported (*70*).

The direct physiological relevance of this reactivation process in the purine auxotrophic *Leishmania* has not been explained yet. Despite this, it is worth mentioning that during transformation of this parasite from the promastigote to the amastigote, activities of a large number of proteins and enzymes, including AdK and several other purine salvage enzymes, are either up- or downregulated (*18*, *19*, *26*). Whether the observed increased activity of AdK in the amastigotes of the parasite is associated with concomitant induction of a new CyP in the cytosol of the amastigotes, as seen during stage-specific alteration of *Plasmodium falciparum* (*71*), however, remains to be investigated. Nevertheless, since CyP, in general, appears to reactivate AdK from all sources by withdrawing ADP aggregation, it would also be of general interest to see whether this type of regulation is instrumental in controlling Ado uptake and its release in higher eukaryotes.

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