A Single-domain Cyclophilin from *Leishmania donovani* Reactivates Soluble Aggregates of Adenosine Kinase by Isomerase-independent Chaperone Function*

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Disaggregation and reactivation of aggregated proteins by chaperones is well established. However, little is known regarding such kind of function of single-domain small cyclophilins (CyPs). Here we demonstrate that, with increasing concentrations, fully active adenosine kinase (AdK) of *Leishmania donovani* tends to form soluble aggregates, resulting in inactivation. Using this inactive enzyme as the substrate, it is shown that a CyP from *L. donovani* (LdCyP) alone can cause complete disaggregation, leading to reactivation of the enzyme. The reactivating ability of LdCyP remains unaffected even in the presence of cyclosporin A and macromolecular crowding agents. The reactivation occurs noncatastytically and is reversible. A truncated LdCyP, devoid of 88 amino acids from the N terminus, is found to be required in near stoichiometric proportion to reactivate AdK, suggesting essentiality of the C-terminal region. Gel filtration and light-scattering experiments together with protein cross-linking studies revealed that both full-length LdCyP and the truncated form directly interact with AdK and convert oligomeric forms of the enzyme to monomeric state. Homology modeling studies suggest that the exposed hydrophobic residues of LdCyP, by interacting with solvent-accessible hydrophobic surface of AdK, pull apart its aggregated inactive oligomers to functional monomers. Clearly, the results are consistent with the interpretation that the higher efficiency of the truncated LdCyP is most likely due to increased exposure of the hydrophobic residues on its surface. These observations, besides establishing *L. donovani* AdK as one of the model enzymes to study aggregation-disaggregation of proteins, raise the possibility that single-domain small CyPs, under physiological conditions, may regulate the activity of aggregation-prone proteins by ensuring their disaggregation.

The mechanism of protein folding *in vitro* is presently studied with a view to understand how proteins fold and remain active inside cells. Broadly, two *in vitro* approaches are followed to study the process of protein folding. The first approach involves prevention of aggregation of proteins by a variety of molecular chaperones during the process of refolding of chemically induced denatured proteins, whereas the second approach explains the kinetics of resolubilization and refolding of heat-treated artificial protein aggregates in the presence of such chaperones (1–4). These elegant studies conclude that during the process of folding, these groups of chaperones selectively recognize and bind with nonnative unstable protein folding intermediates mostly through exposed hydrophobic surfaces and thereby prevent aggregation (see Refs. 5 and 6 and references therein). Whereas these approaches under well defined *in vitro* conditions have provided us with an enormous amount of information about the mechanism of protein folding, the question of how protein aggregates, formed inside cells under various stress conditions, are disaggregated and reactivated within the complex cellular environment is still obscure (7, 8). For instance, the major difference between the well defined *in vitro* condition and those faced inside cells is that the cellular environment is highly complex in terms of the number and concentrations of various soluble and insoluble macromolecules present (9, 10). This situation, known as a “crowded environment,” creates a significant reduction in intracellular space for macromolecules, resulting in an increase in association constants of macromolecules by several orders of magnitude (11, 12).

Cyclophilins (CyPs), a multigenic family of ubiquitous proteins carrying peptidyl-prolyl *cis*-trans-isomerase (PPIase) activity, was originally discovered as a specific receptor of the immunosuppressive drug, cyclosporin A (CsA) (13). It has been shown that the formation of a CyP/CsA complex is an essential step for immunosuppressive effect of the drug (14). Subsequent studies revealed that apart from mediating in the CsA-induced immunosuppressive effect, CyPs are involved in various cellular processes including protein-protein interaction, cell division, acceleration of protein folding, receptor maturation, and several other mechanisms (15–19). Alteration of activities of enzymes and transcription factors also occurs following complexation with CyPs (20, 21). It has been suggested that whereas multidomain large CyPs can function as chaperones, the small single-domain CyPs are restricted only to their PPIase-associated functions (22).

*Leishmania donovani*, a purine auxotrophic and dimorphic...
protozoan parasite, is the causative agent of kala azar in human. This protozoon exists as a flagellated promastigote (extracellular form) in the sand fly vector and transforms into amastigote (intracellular form) in the mammalian macrophages. Due to purine auxotrophy, this parasite utilizes host purines using its purine salvage pathway enzymes. Adenosine kinase (AdK), which catalyzes phosphorylation of Ado to AMP, is one of the key purine salvage pathway enzymes of this parasite. Because of its unique biochemical properties and stage-specific differential activity patterns, this enzyme is considered as one of the prospective drug-targeting sites (see Ref. 23, references therein, and Ref. 24). One of the major activities of this laboratory centers around studies related to structure-function analysis of this enzyme (25–27). During kinetic analysis, it was unexpectedly noticed that the purified AdK, at higher concentrations, ceases to follow linear kinetics. While investigating the possible cause of this inactivation, we discovered that AdK, which is fully active under dilute conditions, to near homogeneity using a nickel-nitrilotriacetic acid-agarose column as per the manufacturer’s suggested procedure. For all biochemical experiments, the purified proteins were extensively dialyzed against a buffer containing 20 mM Tris, pH 7.5, 1% glycerol, and 1 mM dithiothreitol was used.

Construction and Expression of Full-length and Deleted LdCyP—Full-length recombinant LdCyP was expressed and purified using procedures described previously (28). To construct the N-terminal deleted clone of LdCyP (N_{22-DEL}), the coding sequence (amino acids 89–187) was amplified by PCR and ligated in-frame at the BamHI/SalI sites of the expression vector pQE30 (Qiagen). The 5′-PCR primer (sense), 5′-CGACCAAGATTGCACATCTCAACACCTTGATG-3′ with a BamHI site and 3′-primer (antisense), 5′-CGACCTCTGAGTCAGCTCCCTCCTTCTT CTC-3′ containing a Xhol site were used. Using the similar strategy, three C-terminal deleted fragments (viz. C_{96-157}-DEL, C_{142-157}-DEL, and C_{158-157}-DEL) were ligated at the BamHI/SalI sites of the expression vector pQE32. E. coli M15 cells harboring either plasmid, was amplified by PCR and ligated in-frame at the NdeI sites of pQE30 expression vector.

M15 cells harboring the plasmid was purified to near homogeneity using a nickel-nitrilotriacetic acid-agarose column (30) observed that the CyP acts as chaperone (18, 30). In those experiments, using guanidine HCl-treated human carbonic anhydrase, Freskgard et al. (18) suggested that the chaperone function of CyP is exerted only at the early folding intermediate state, thereby preventing off-pathway reaction, and the isomerase activity is performed later in the folding process, whereas Ou et al. (30) observed that the ability of the inactive PPIase to effect reactivation of denatured creatine kinase and prevent its aggregation during folding was weaker than that of active PPIase. In other words, most of the studies on chaperone function of CyP described so far, dealt with prevention or suppression of aggregation of the early steps of protein folding. However, these results never addressed whether small single-domain CyPs could rescue functional monomers from already aggregated inactive proteins. Our experiments demonstrated the ability of LdCyP to reactivate such a type of soluble aggregates of proteins in vivo is therefore clearly unique and may reflect the capacity of CyPs to activate such a type of soluble aggregates of proteins interfered with by Rayleigh scattering, and K is the constant that depends on the refractive index of the solvent. Assuming partial specific volume (\(\bar{\rho}\)) of all globular proteins to be same, the equation further simplifies to the following:

\[ R_s = K' \cdot c \cdot r_s^3 \]  

(2)

where \( R_s \) represents Stokes radius, and \( K' \) is a new constant. When proteins are noninteracting, the scattering becomes directly proportional to the concentration of proteins and their Stokes radii. However, a deviation from linearity with increasing concentra-
tions of the protein provides an indication of association-dissociation
(32, 33). Scattering was measured in a Hitachi F-4500 spectrofluorometer at 25 °C. The excitation and emission wavelengths were fixed at 350 nm, where AdK and LdCyP had no absorption. Slit widths were 5 nm. Scattering of samples was followed for 5 min, and minimum values recorded for each sample were considered.

Modeling Studies—Predictions of the three-dimensional structures of AdK, full-length LdCyP, and its truncated version were made by knowledge-based homology modeling using InsightII 98.0 of MSI (Bio-
sym Technologies, San Diego, CA), ABGEN (34), and our in house package of MODELYN and ANALYN (35) in UNIX as well as in the Microsoft Windows environment. Energy minimization and molecular dynamics were performed with the InsightII 98.0/Discover package using the cff91 force field on a Silicon Graphics OCTANE work station. Energy minimizations were done with a convergence criterion of 0.001 kcal/mol, using a combination of steepest descent and conjugate gradient methods (100 steps each). Molecular dynamics simulations were performed using a time step of 1 fs for 100 steps of equilibration and 1000 steps of dynamics. In the case of truncated LdCyP, distance constraints were applied to the other parts of the molecule while running minimization and dynamics for regularization of selected segments. Repeated steps of molecular dynamics, selection of conformation with least potential energy, and energy minimization were performed until satisfactory conformational parameters were obtained. The electrostatic potential surfaces of the protein models were determined by MOLMOL (36). Protein BLAST (37) was used for searching the homologous proteins of known structures taking AdK/LdCyP as the query sequence in the Protein Data Bank data base. ClustalW (38) was used for multiple alignments of protein sequences. PROCHECK (39) was used for checking the structural parameters and comparing with refer-
ence protein structures.

RESULTS

AdK-mediated Phosphorylation of Ado in the Presence of LdCyP—In the presence of saturating substrates, most en-
yzymes under permissible assay conditions follow time-depend-
ent linear reaction kinetics with increasing protein concentra-
tions. The purified *L. donovani* AdK, however, was found to behave anomalously in this respect. At a comparatively low concentration of the enzyme, the rate of Ado phosphorylation, as expected, followed a linear kinetics. However, at higher concentrations of the protein, the reactions ceased to follow concentration and time-dependent linear reaction and became increasingly sluggish with time (Fig. 1). The presence of LdCyP in the mixture clearly prevented this trend and allowed the reactions to proceed faster as evident from the increase in the rate of reaction and extent of AMP formation (Fig. 1, inset). The reactivation of AdK was not affected even when CsA was added in molar excess over LdCyP, indicating that the process is isomerase-independent. These results suggest that the observed sluggish rate of the reaction at higher concentrations of AdK was clearly not due to substrate limitation. The kinetic nature of the reaction remained identical even if the reaction was initiated with ATP, indicating that ATP did not have any role in the activation process (data not presented). Instead of LdCyP, BSA and other proteins like lysozyme and histone, even if added in a 20–400-fold excess, did not exert any stimu-
laratory effect on AdK-mediated phosphorylation (Table I). Sor-
bitol, an osmolyte, also failed to reactivate the enzyme. Ficoll 70 and BSA are well known to mimic the in vivo

| Experiment | AdK activity (nmol AMP/mg/min) |
|------------|--------------------------------|
| AdK        | 125                            |
| AdK + BSA  | 137                            |
| AdK + histone | 143                         |
| AdK + lysozyme | 142                       |
| AdK + sorbitol | 126                        |
| AdK + Ficoll 70 | 126                        |
| AdK + LdCyP | 501                            |
| AdK + Ficoll 70 + LdCyP | 512                        |
| AdK + BSA + LdCyP | 505                        |
| AdK + KCl (1.2 M) | 125                        |
| AdK + KCl (1.2 M) + LdCyP | 125                        |

*Table I: Effects of various agents and LdCyP on the activity of AdK.*

The activity of AdK (10 nM) was measured at 30°C after 30 min. Wherever indicated, Ficoll 70 (72 mg/ml), BSA (80 mg/ml), histone (4 mg/ml), lysozyme (4 mg/ml), and sorbitol (250 mM) were included in the reaction mixture. LdCyP concentration was maintained at 200 μg/ml.
low AdK concentrations (physiologically suboptimal), all of the reactions, as expected of saturation kinetics, reached various levels of steady states with a molar excess of LdCyP, indicating that complete reactivation of the enzyme can indeed be attained, whereas, at high concentrations of AdK, the reactivation was manifested at concentrations of LdCyP close to 1:1 molar stoichiometry. However, due to the limitation of the assay system used here, it was not possible to determine the exact stoichiometry. Nevertheless, from these results, it is clear that the interaction of LdCyP with AdK that results in activation of the enzyme is probably governed by the product of active masses of the two reactants. Perhaps the large requirement for LdCyP at a low concentration of AdK was due to low probability of collision between AdK and LdCyP. Taken together, observations made in Figs. 1 and 2 led us to conclude that the action of LdCyP occurs most likely through protein-protein interaction rather than CsA-sensitive cis-trans isomerization.

**Sustained Presence of LdCyP Is Essential for Optimal Activity of AdK**—In order to gain an understanding as to how LdCyP leads to reactivation of AdK, we followed the effect of adding LdCyP at different time intervals during the progress of Ado phosphorylation (Fig. 3). Although the concentration of LdCyP chosen for this purpose was in molar excess over AdK, it was suboptimal with respect to its requirement for maximal stimulation of AdK present in the reaction mixture (Fig. 2). In the first step, three separate sets of phosphorylation reactions in the absence (reaction C) and in the presence of 5 nM (reaction B) and 22 μM (reaction A) LdCyP were started simultaneously using a fixed amount (50 nM) of AdK in each reaction. While the reaction containing a high molar excess of LdCyP (22 μM) was allowed to proceed uninterrupted for 120 min (reaction A), the other two sets, after a period of 30 min, were divided into two equal halves (reactions B and C). The incubation of all four subdivided reactions was further continued for 120 min with the difference that one of the subdivided reactions from each set received an additional dose of LdCyP that raised its final concentration to 22 μM. At the indicated time intervals, samples were withdrawn and processed for AMP formation. As expected, the reaction without LdCyP, after the initial few minutes, became sluggish (reaction C). In contrast, the other two sets of reactions (reactions A and B), depending upon the amount of LdCyP present, progressed with higher initial rates, resulting in increased yield of AMP. An additional dose of LdCyP to reactions B and C at the 30th min resulted in a further increase in the reaction rate. Interestingly, the increased rate, as evident from the progress of the reaction and level of AMP formation, was nearly comparable with the reaction progressing in parallel from the beginning in the presence of a high molar excess of LdCyP (reaction A). Since reaction B was initiated with a low molar excess of LdCyP, the stimulation observed with the second booster dose of LdCyP is consistent with the interpretation that the amount of LdCyP added initially in the assay was not sufficient to reanimate AdK completely. Taken together, the nature of the progress curves of all three reactions provides a compelling rationale for the interpretation that a continuous presence of optimal amount of LdCyP is needed for sustained activity of AdK. The failure to observe any lag even at the early stages of the reaction also implied that the reactivation process is extremely rapid to be detected under the present assay condition. A similar conclusion was also made while studying the reactivation of RepA protein by ClpA chaperone (41).

**LdCyP Disaggregates Oligomeric AdK**—Having confirmed that the stimulatory effect of LdCyP on AdK was not due to generalized change of protein environment, we set out to analyze the nature of the effect that LdCyP exerts. Given the present scenario, two most obvious reasons (viz. improper conformation or simple aggregation) can cause inactivation of the enzyme. To investigate this, a concentrated preparation of purified AdK was subjected to gel filtration using HPLC. Results shown in Fig. 4 indicate that the enzyme alone eluted over a wide range of molecular mass, with a substantial portion of the loaded protein eluting out at positions much heavier than the monomeric AdK (38 kDa). This type of elution pattern is most likely due to oligomerization of the protein. Interestingly, the addition of LdCyP in a 1:1 molar proportion dramatically changed the elution profile of AdK, resulting in the appearance of the protein at position expected of monomeric enzyme. These results strongly support the contention that in the presence of LdCyP, oligomeric AdK molecules get dissociated to their monomeric form. ATP was not needed in this process. In order to confirm and analyze the type of oligomers formed, the prepa-
Determination of interaction between AdK and LdCyP by a light-scattering experiment. Scattering intensities of AdK (0.5 and 2 μM), LdCyP (0.5 and 2 μM), and a mixture of AdK and LdCyP were determined. □, ■, and ▼, LdCyP, AdK, and a mixture of LdCyP and AdK, respectively; ▼, additive theoretical value of the LdCyP/AdK mixture. Experiments represented in A and B were carried out in the presence of 0.5 and 2 μM of LdCyP, respectively, keeping the concentration of AdK fixed at 0.5 μM, whereas the concentrations of both AdK and LdCyP in the experiment described in C were maintained at 2 μM.

**Fig. 4.** Gel filtration chromatography of AdK before and after treatment with LdCyP. The solid line shows the elution profile of the enzyme prior to LdCyP treatment, whereas the dotted line depicts its profile after exposure to LdCyP. Details are described under “Experimental Procedures.” The inset shows PAGE of purified AdK (10 μg) run in the presence and absence of SDS and stained with Coomassie Blue.

**Fig. 5.** Demonstration of interaction between AdK and LdCyP by a light-scattering experiment. Scattering intensities of AdK (0.5 and 2 μM), LdCyP (0.5 and 2 μM), and a mixture of AdK and LdCyP were determined. □, ■, and ▼, LdCyP, AdK, and a mixture of LdCyP and AdK, respectively; ▼, additive theoretical value of the LdCyP/AdK mixture. Experiments represented in A and B were carried out in the presence of 0.5 and 2 μM of LdCyP, respectively, keeping the concentration of AdK fixed at 0.5 μM, whereas the concentrations of both AdK and LdCyP in the experiment described in C were maintained at 2 μM.

**Fig. 6.** Cross-linking of AdK with LdCyP in the presence of DSS. AdK (11 μg) was incubated with DSS in the presence of increasing concentrations (1:1, 1:2, and 1:4 molar proportions) of LdCyP (lanes 4–6 and 9–11). The reaction mixtures were run on SDS-PAGE (13%) and immunoblotted with polyclonal antibodies raised against both AdK (lanes 2–6) and LdCyP (lanes 7–11). In lanes 2 and 7, standard AdK and LdCyP were loaded, whereas lanes 3 and 8 contained AdK and LdCyP samples that were incubated with DSS in the absence of LdCyP and AdK, respectively. Lane 1 shows the positions of marker proteins. Details are described under “Experimental Procedures.”

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**LdCyP Directly Interacts with AdK**—To demonstrate a direct interaction between AdK and LdCyP, light scattering of fixed amounts AdK both in the presence and absence of LdCyP was carried out (Fig. 5). LdCyP, as expected of a noninteracting globular protein, produced linear increase in scattering intensity with increasing concentrations of the protein. In contrast, the scattering intensity of increasing concentrations of AdK failed to maintain the same linearity (Fig. 5, B and C, black bars). This is clearly due to aggregation (see “Experimental Procedures”). Interestingly, the experimental scattering values of the AdK and LdCyP mixture (segmented bars) fell far short of their theoretically calculated additive scattering values (hatched bars). A mixture of equimolar concentrations of AdK and BSA, however, produced additive scattering intensity, as expected of noninteracting globular proteins (data not shown). These results are explainable only when a direct interaction between AdK and LdCyP is considered.

Despite the above result, it was not possible to demonstrate direct complex formation between LdCyP and AdK on a nickel-nitrilotriacetic acid column, suggesting the interaction to be extremely weak or transient. The complex was, however, clearly visible when the two proteins were incubated in the presence of DSS (Fig. 6). Incubation of a fixed amount of AdK with increasing concentrations of LdCyP in the presence of DSS resulted in gradual appearance of several low molecular weight AdK antibody-reactive bands of different molecular masses with concomitant disappearance of the largest multimeric AdK aggregate found at the top of the gel (lanes 3–6). An examination of these bands indicated that their molecular masses were close to different oligomers of monomeric AdK (38 kDa). The notable aspect of this experiment is the gradual appearance of a new band with a molecular mass corresponding to 56 kDa. Clearly, the 56-kDa molecular mass band corresponds to the combined mass of AdK and LdCyP. Cross-reactivity of the 56-kDa band with LdCyP antibody reaffirmed that the band was indeed the complex of LdCyP and AdK in a 1:1 molar proportion (lanes 8–11).

**C-terminal Region of LdCyP Is Sufficient for Reactivation**—Multidomain large CyP such as CyP40 and other immunophi-

lin-like FKBP-52 have been shown to exhibit chaperone-like function (42, 43). Results presented here clearly indicate that LdCyP, despite being a single-domain small CyP, could disaggregate and reactivate AdK. Therefore, it was of interest to dissect out the region responsible for the chaperone-like function of LdCyP. In order to investigate this, we designed a truncated clone (N22–ss-DEL) of LdCyP that lacked the first 88 amino acids from the N-terminal end (Fig. 7A). Both the full-length LdCyP and N22–ss-DEL peptide were expressed and purified to near homogeneity (Fig. 7B, inset). Interestingly, the
effect of the N22-88-DEL peptide fragment, which did not have any detectable [3H]Ca2+ binding activity as measured by Sephadex-LH column binding assay (data not shown), on AdK reactivation was dramatic (Fig. 7). Results show that the amount of the peptide fragment required for maximal stimulation of the same amount of AdK was much less than the full-length LdCyP and approached mol/mol stoichiometry. High concentrations of Ficoll 70 did not affect the reactivation process. The interaction was also evident when N22-88-DEL peptide was used instead of LdCyP in the light scattering experiment described in the legend to Fig. 5 (data not shown). These findings suggest that the C-terminal region is sufficient for reactivation of the enzyme.

Reversibility of AdK Reactivation—To investigate this, oligomeric AdK was first reactivated by the addition of LdCyP. The mixture was loaded onto a 5'-AMP-agarose column, and the column was washed thoroughly according to the published procedure (23). Under this condition, LdCyP comes out in the flow-through and wash fractions, but the kinase activity remained attached to the column. AdK was subsequently eluted from the column with buffer containing 1 mM Ado and assayed for the activity. Results presented in Table II show that, as anticipated, the addition of LdCyP to AdK resulted in increase in the specific activity of the enzyme. However, the specific activity of the enzyme after elution from the column was comparable with the observed specific activity prior to the addition of LdCyP. Interestingly, the addition of exogenous LdCyP in the same molar proportion to the column-eluted enzyme again led to reactivation. When LdCyP was replaced with a much lower concentration of N22-88-DEL in an identical experiment, a similar result was obtained. These observations are therefore consistent with the interpretation that reactivation of AdK by LdCyP or its truncated form is reversible and does not involve permanent alteration of enzyme conformation.

N22-88-DEL LdCyP Is More Efficient than the Full-length LdCyP in Converting Oligomeric AdK to Monomers—Experiments described in Fig. 7 and Table II indicated that N22-88-DEL peptide was severalfold more efficient than LdCyP in reactivating same amount of AdK. To investigate this further, a fixed amount of AdK was allowed to interact separately with both N22-88-DEL peptide and LdCyP in the presence of DSS. A fixed amount of the above mixtures from each reaction was electrophoretically fractionated on SDS-PAGE (10%) and immunoblotted with AdK antibodies (data not shown). The most interesting aspect of the experiment is the demonstration of substantial conversion of the loaded AdK in monomeric form by both versions of LdCyP (Fig. 8, lanes 3 and 4). Control experiments consisting of equal amounts of AdK both in the presence and absence of DSS were also included (lanes 1 and 2). Following incubation, fixed amount of the mixture from all four reactions were electrophoretically fractionated on SDS-polyacrylamide gel (10%) and immunoblotted with AdK antibodies. Results showed that the full-length LdCyP, N22-88-DEL peptide formed an adduct with AdK. This is evident from the appearance of a new immunopositive band at a position corresponding to a molecular mass of around 49 kDa (Fig. 8, lane 3), smaller than the LdCyP-AdK complex (Fig. 8, lane 4). The 49-kDa band was also detected when another identically run gel was immunoblotted with LdCyP antibodies (data not shown). The most interesting aspect of the experiment is the demonstration of substantial conversion of the loaded AdK in monomeric form by both versions of LdCyP (Fig. 8, lanes 3 and 4). The amount of N22-88-DEL required for monomerization of the same amount of AdK is clearly lower as compared with the full-length LdCyP. These

Fig. 7. The C-terminal region of LdCyP is responsible for reactivation of AdK. A, schematic representation of full-length LdCyP and N22-88-DEL fragment. Amino acid numbers define the domain boundaries. The two most conserved motifs present in most CyPs, depicted by dark and hatched boxes, are shown. B, requirements of truncated (△) and full-length (○) LdCyP were compared for stimulation of a fixed amount of AdK (50 nm) in the presence (△) and in the absence (○) of Ficoll 70. The reactions were carried for 30 min. Insets, Coomassie Blue-stained bands of purified recombinant full-length LdCyP (a) and N22-88-DEL fragment (b).

| AdK activity | Experiment | Specific activity |
|--------------|-------------|------------------|
|              | nmol AMP/mg min |
| Before 5′-AMP column | AdK | 164 |
| | AdK + LdCyP | 420 |
| | AdK + N22-88-DEL | 500 |
| Post 5′-AMP column | AdK | 146 |
| | AdK + LdCyP | 500 |
| | AdK + N22-88-DEL* | 740 |

* LdCyP or N22-88-DEL, in the same molar proportion, was added exogenously prior to initiation of the reaction. Assay was for 30 min at 30 °C.
results are consistent with the interpretation that N terminus-truncated LdCyP acts as better chaperone than the full-length LdCyP in reactivating AdK. Further, it is to be noted that unlike previous studies, which implicated involvement of the LdCyP in reactivating AdK. Further, it is to be noted that truncated LdCyP acts as better chaperone than the full-length results are consistent with the interpretation that N terminus-sequence (28), is capable of interacting with AdK.

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DISCUSSION

Original models depicting the function of chaperones were described by their ability to prevent aggregation of protein substrates by binding to their nonnative early folding intermediates and assisting them in reaching a functional conformation (44). In fact, prevention or suppression of aggregation during refolding of urea or guanidine HCl-denatured proteins was indeed considered to be the most important aspect in the chaperone-facilitated folding of proteins. Toward this goal, several chaperones were reported, among which CyP was considered to be one of the candidates (18, 20, 30). However, to the best of our knowledge, no experimental evidence existed to support the notion that single-domain small CyPs can rescue functional monomers from aggregates of inactive enzymes and/or proteins. In this paper, two major novel findings are reported. First, we serendipitously discovered that adenosine kinase of L. donovani tends to form soluble aggregates at higher concentrations, resulting in its inactivation; second, LdCyP, a small single-domain CyP from L. donovani, is capable of disaggregating this inactive enzyme to functionally active monomers in a Ca2+ as well as ATP-independent manner. This astonishing observation compelled us to analyze the mechanism of aggregation and to think about its remedial measures. The property of LdCyP described here, to a certain extent, is comparatively less under normal in vivo conditions, we are tempted to suggest that the disaggregating property of LdCyP observed here could be physiologically relevant. In terms of ATP requirement, LdCyP seems to belong to the class of ATP-independent chaperones such as FKBP-52, p23, and a few others but differs with respect to the nature of protein aggregates on which it acts (see Ref. 43 and references therein).

A large body of evidence suggests that the PPIase activity of immunophilins is responsible for various functions (16, 46, 47). However, the controversy regarding the chaperone function of small immunophilins has yet to be fully settled (18, 20, 48). It was suggested that only multidomain large immunophilins can exert chaperone-like function via their additional domain, whereas abundant single-domain small immunophilins are restricted to only PPIase activity (20). The fact that CyPA and nina A forms stable complexes with their protein substrates, human immunodeficiency virus Gag1 polyprotein and Rh1 rhodopsin, respectively, is well established (19, 40). However, the interaction of CyPA with human immunodeficiency virus Gag1 protein does not lead to any increase in the yield of fully assembled virus particles, suggesting that it is unlikely that CyPA is chaperoning the folding of that protein (49, 50). Moreover, nina A of Drosophila, which is also required in quantitative amounts for maturation of Rh1, has been shown to escort its protein substrate through the secretory pathway (19). Our results suggest that LdCyP disaggregates and reactivates proteins with extreme rapidity, and the process is independent of its PPIase activity. This property of LdCyP thus supports the most widely accepted definition of chaperones that are expected to transiently bind to inactive conformers of another protein, thus facilitating their disaggregation (51). A recent result, which showed PPIase-independent chaperone function of a small periplasmic protein, FkpA, of E. coli, is similar to our observation (52).

Our studies indicate that the mechanism of reactivation of AdK by LdCyP follows the principles of physicochemical equilibrium rather than enzymatic process. This is supported by the fact that the amount of LdCyP required for increased reactivation of the enzyme followed an inverse relationship with the amount of AdK present in the reaction mixture. The molar excess of LdCyP required for optimal reactivation of physiologically suboptimal concentrations of AdK was perhaps due to low probability of interaction between LdCyP and AdK. Interestingly, however, when 88 amino acids from the N-terminal end of the intact protein was deleted, the concentration of the N22-88-DEL peptide fragment needed for the same level of activation was drastically reduced and approached a value close to molar stoichiometry. Therefore, our ability to detect the formation of AdK-N22-88-DEL adduct at low AdK/N22-88-DEL
peptide ratio with concomitant conversion of most of the oligomeric AdK to monomeric form is consistent with the explanation that the extent of reactivation of AdK would be dependent upon the efficiency of LdCyP to convert oligomeric AdK to its monomeric form. In this context, it may be worth pointing out that the cysteine residue of CyP from porcine kidney was implicated in binding of peptide substrate (30). However, our experiments with truncated LdCyP, which does not harbor any cysteine (28), show that this is not applicable in the present case. These results may indicate that the C-terminal region of the full-length LdCyP, responsible for binding with AdK, was probably not fully accessible due to masking. The fact that the chaperone activity resides in the C-terminal region has also been demonstrated using mammalian FKBP-52 and FK 506-binding protein from thermophilic archaeon (53, 54).

What structural characteristics make the truncated LdCyP more efficient than the full-length LdCyP in reactivating AdK? Is it simply due to the fact that the C-terminal region of the full-length LdCyP is not fully accessible for interaction with AdK or for any other reasons? In an attempt to address this issue, LdCyP and its truncated form were modeled along with AdK, using coordinates available from the crystal structures of their human counterparts (see Ref. 28, references therein, and Ref. 55). AdK from all sources studied so far is a monomeric molecule. The crystal structure of the human enzyme showed that it is composed of two unequal sized domains with no amino acid homology between them (55). The Ado-binding site has been shown to reside in the cleft between two domains. Adenosine kinase of L. donovani displays 40% homology with human AdK (27). From the model and electrostatic potential mapping of the surface, it is apparent that the parasite enzyme harbors a large number of solvent-exposed hydrophobic residues on the surface of the molecule (Fig. 9). These exposed hydrophobic residues could therefore be instrumental for protein-protein interaction leading to oligomerization. Comparison of similarly designed models of LdCyP and its truncated form (Fig. 10) further indicated that several hydrophobic amino acids (Phe^67, Ile^78, Tyr^79, Phe^83, Phe^112, Ile^114, Ile^129, and Ile^142), which normally remains buried in the full-length LdCyP (Fig. 10, A and B) became more solvent-exposed in the truncated form, thereby providing increased hydrophobic surface for interaction (Fig. 10, C and D).
of the truncated LdCyP can therefore be due to the increased exposure of these hydrophobic residues on the surface of the truncated LdCyP molecule. Similar mechanisms have been proposed to explain the binding specificity of the trigger factor of *E. coli* and FKBP12 (56, 57). Direct verification of this proposal, however, awaits further experiments.

Taken together, these results suggest that LdCyP does not induce global alteration in the conformation of the substrate protein; rather, it facilitates kinetic partitioning between oligomeric and monomeric forms of the enzyme by binding locally to specific hydrophobic sites of the substrate protein responsible for interaction among themselves. Based on this information, we propose a schematic working model for disaggregation and subsequent reactivation of AdK by LdCyP (Fig. 11). This scheme, which is self-explanatory, proposes transient and reversible interaction between LdCyP and oligomeric AdK molecules, leading to disruption of noncovalent intermolecular contacts between aggregated molecules. The proposed mechanism is analogous to ClpA and ClpX chaperones, which are also known for their disaggregating properties (41, 58).

Contact between aggregated molecules, leading to disruption of noncovalent intermolecular contacts among themselves. Based on this information, we propose a schematic working model for disaggregation and subsequent reactivation of AdK by LdCyP (Fig. 11). This scheme, which is self-explanatory, proposes transient and reversible interaction between LdCyP and oligomeric AdK molecules, leading to disruption of noncovalent intermolecular contacts between aggregated molecules. The proposed mechanism is analogous to ClpA and ClpX chaperones, which are also known for their disaggregating properties (41, 58).
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A Single-domain Cyclophilin from *Leishmania donovani* Reactivates Soluble Aggregates of Adenosine Kinase by Isomerase-independent Chaperone Function

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