Isomerase-Independent Chaperone Function of Cyclophilin Ensures Aggregation Prevention of Adenosine Kinase Both *in vitro* and under *in vivo* Conditions†

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ABSTRACT: Using inactive aggregates of adenosine kinase (AdK) from *Leishmania donovani* as the model substrate, we recently demonstrated that a cyclophilin (LdCyP) from the same source in an isomerase-independent fashion reactivated the enzyme *in vitro* by disaggregating its inactive oligomers [Chakraborty *et al.* (2002) *J. Biol. Chem. 277*, 47451−47460]. Besides disrupting preformed aggregates, LdCyP also prevents reaggregation of the newly formed active protein that is generated after productive refolding from its urea-denatured state. To investigate possible physiological implications of such phenomena, a unique expression system that simultaneously induces both AdK and LdCyP in naturally AdK-deficient *Escherichia coli*, was developed. Both *in vitro* and *in vivo* experiments revealed that oligomerization is an inherent property of this particular enzyme. In vivo protein cross-linking studies, activity determination analysis and Ado phosphorylation experiments carried out in cells coexpressing both the proteins unequivocally demonstrated that, similar to the phenomena observed *in vitro*, aggregates of the enzyme formed *in vivo* are able to interact with both LdCyP and its N-terminal truncated form (N_{22-88}DEL LdCyP) in a crowded intracellular environment, resulting in aggregation prevention and reactivation of the enzyme. Our results indicate that the isomerase-independent chaperone function of LdCyP, detected *in vitro*, participates *in vivo* as well to keep aggregation-prone proteins in a monomeric state. Furthermore, analogous to yeast/bacterial two-hybrid systems, development of this simple coexpression system may help in the confirmation of interaction of two proteins under simulated *in vivo* conditions.

The term "molecular chaperone" is attributed to a family of altruistic proteins which by interacting with appropriate targets (proteins/enzymes) enhance their functional efficiency. Most extensively studied chaperones with wide range of functions are the GroEL-GroES and DnaK-DnaJ-GrpE chaperone machineries present in *Escherichia coli.* The essentiality of chaperone proteins, GroEL and GroES, in assembly of subunits and prevention of aggregation during renaturation of proteins are well established (*1*−*3*). DnaK and HSP70 chaperones of *E. coli* have also been reported to interact with protein substrates in conjunction with DnaJ, GrpE, and in some cases with ClpB cochaperones to carry out folding, remodeling, assembly, and disassembly of multisubunit protein complexes, as well as disaggregation of previously aggregated protein molecules (*4*−*10*). Moreover, there are also reports in the literature to indicate the presence of protein-specific chaperones (*11*, *12*).

Cyclophilins (CyPs), a class of ubiquitous proteins displaying peptidyl-prolyl *cis*−*trans* isomerase (PPIase)[1] activity, were originally identified as the specific receptor of the immunosuppressive drug cyclosporin A (CsA) (*13*). Subsequently, it was discovered that CyPs are also involved in various cellular processes including protein−protein interaction, facilitation of protein folding, receptor maturation, and several other mechanisms (*14*−*23*).

Leishmania donovani, a purine auxotrophic flagellated parasitic protozoon, causes kala-azar in humans. The organism has a digenetic life cycle. This parasite exists as a promastigote (extracellular form) in the sand fly vector but is converted into an amastigote (intracellular form) upon entry into mammalian macrophages. During this transformation process, the activity of a large number of proteins has been reported to be stage-specifically altered (*24*−*26*). The activity of adenosine kinase (AdK), one of the purine salvage enzymes of this parasite catalyzing phosphorylation of Ado to AMP, has been shown to display several fold higher activity upon transformation into amastigotes (*27*). However, to date, no evidence exists to indicate that the higher activity observed is due to increased protein synthesis.

We recently discovered that a CyP (LdCyP) from this parasitic protozoon possesses an isomerase-independent chaperone function by which it can reactivate inactive aggregates of the protein AdK (*28*). The uniqueness of this

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[1] Abbreviations: LdCyP, *Leishmania donovani* cyclophilin; PPIase, peptidyl-prolyl *cis*−*trans* isomerase; CsA, cyclosporin A; AdK, adenosine kinase; DSS, disuccinimidyl suberate; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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study lies not only in the disaggregation process but also in the characteristics of the AdK aggregates that have been used here as the substrate. In general, model proteins that are mostly used as substrates to study the chaperone function of proteins are known not to be present in the aggregated state under physiological conditions. Instead, they are artificially converted into aggregates (*2*−*4*, *8*, *29*). Therefore, direct extrapolation of these results within the complex cellular environment becomes extremely difficult in majority of the cases. Moreover, the questions as to whether (i) aggregate formation takes place during or after folding of proteins *in vivo* or (ii) aggregate formation is prevented or aggregates formed are disaggregated and subsequently reactivated by chaperones inside a crowded cellular environment remain ill-explored (*30*, *31*).

Because a proper understanding of these phenomena has important bearing with regard to regulation of functional activities of enzymes, proteins, or both and their secretion from cells or organelles, a detailed study on these aspects appears essential. Our observations on the inherent tendency of AdK to form soluble aggregates and its disaggregation and reactivation by LdCyP, which clearly is a deviation from the so-far described chaperone functions, was therefore exploited to investigate the physiological implications of this phenomenon.

By developing a novel coexpression system, we, in this report, present experiments to show that under a physiological environment, aggregates of AdK formed can be dissociated by LdCyP to form active enzyme. Furthermore, we have presented direct evidence to show interaction of AdK with LdCyP in the crowded cellular environment, suggesting a potential role of CyP proteins functioning as *in vivo* chaperones.

MATERIALS AND METHODS

Materials. All chemicals used were of analytical grade. [2-^{3}H]-Adenosine (23 Ci/mmol) was obtained from Amersham-Pharmacia Biotech. CsA was generously provided by Dr. G. Engel of Sandoz Pharmaceuticals, Basel, Switzerland. Disuccinimidyl suberate (DSS), a noncleavable, amine-reactive, homobifunctional cross-linker, was purchased from Pierce.

Expression and Purification of AdK. Construction of the AdK-expressing clone has been described in our previous publication (*28*). For all the biochemical experiments, imidazole-eluted His-tagged AdK extensively dialyzed against a buffer containing 20 mM Tris, pH 7.5, 1% glycerol, and 1 mM DTT was used.

Purification of Recombinant LdCyP and Its Truncated Version. LdCyP and its truncated version (N_{22-88}DEL LdCyP) were purified from IPTG-induced *E. coli* M15 strain harboring plasmids containing the respective encoding sequences, following the procedure described previously (*28*).

Adenosine Kinase Assays and Other Methodologies. AdK assays under saturating substrate concentrations were carried out following the procedure described previously (*28*). AdK activity in the crude extract was determined according to the standard procedure (*32*). Other methodologies have been described elsewhere (*33*−*35*).

Denaturation of AdK and Reactivation Assays. Denaturation of AdK (5 μM) was carried out by incubating the enzyme at 30 °C for 1 h in the presence of 6 M urea. AdK did not show any loss of activity up to 2 M urea but was completely inactivated following exposure to 6 M urea (data not shown). To follow activity during refolding, the completely denatured enzyme was directly assayed by adding to the enzyme assay mixture in a proportion that brought down the enzyme and urea concentrations to 50 nM and 60 mM, respectively, while to determine the refolding yield, the denatured enzyme was first diluted 20-fold with reactivation buffer (20 mM Tris, pH 7.5, 1 mM DTT, and 1% glycerol). Wherever indicated, chaperone was included in the reactivation buffer. Immediately after dilution, aliquots (10 μl) were quickly withdrawn at various time intervals and added to the assay mixture to make it 50 μL. The time of incubation was 2 min. This short-time incubation completely eliminated the problem of protein aggregation, an inherent property of AdK (*28*). The percent of reactivated enzyme formed was determined relative to the activity of the same amount of native enzyme. Apparent rate constant was determined using first-order kinetics.

Fluorescence Measurements. Intrinsic tryptophan fluorescence of AdK was measured at room temperature using a Hitachi F-4500 fluorescence polarization spectrophotometer. Following excitation at 295 nm, the emission was monitored between 300 and 400 nm. The slits for both excitation and emission were set at 5 nm, and correction for inner filter effect ($A_{295} > 0.1$) was calculated using the formula $F = F_{obs}$ antilog$[(A_{exc} + A_{em})/2] \times$ dilution factor, where A_{exc} and A_{em} are absorbance at excitation and emission wavelengths, respectively. Denaturation of AdK (25 μM) was carried out by diluting the enzyme 25-fold in refolding buffer supplemented with 6 M urea. During renaturation, the urea-denatured enzyme (25 μM) was quickly diluted 25-fold with refolding buffer.

Densitometric Estimation of AdK. Known volumes of crude extracts from various cell clones expressing AdK were loaded on SDS−PAGE (10%) and immunoblotted with anti-AdK antibodies. Bands visualized were subjected to densitometric scanning and the concentration of AdK present in respective crude extracts was determined after comparison with known standard recombinant AdK run parallely as control.

Cross-Linking Studies. In vitro cross-linking between AdK and LdCyP or its truncated version was carried out in the presence of DSS following the methodology described earlier (*28*). The extent of cross-linking during renaturation of the denatured AdK (40 μg/ml) was monitored by incubating the enzyme with the cross-linker for different time periods either in the absence or in the presence of LdCyP (3 μM). A control experiment was performed in the same manner using the same concentration of native AdK. At each time period (5, 15, 30, and 60 min), aliquots were withdrawn from the renaturation mixture, run on SDS−PAGE (10%), and visualized following immunoblotting with polyclonal antisera raised against AdK (*36*).

For cross-linking in crude extracts, crude protein containing the equivalent of 5 μg of AdK measured by densitometric scanning was incubated with 0.5 mM DSS for 30 min at 30 °C. After complete reaction, samples were analyzed as above.

To carry out intracellular cross-linking, cells grown up to $OD_{595} \approx 0.8$ were exposed to DSS (0.5 mM) for 30 min prior to IPTG induction. IPTG (1 mM) was added to the

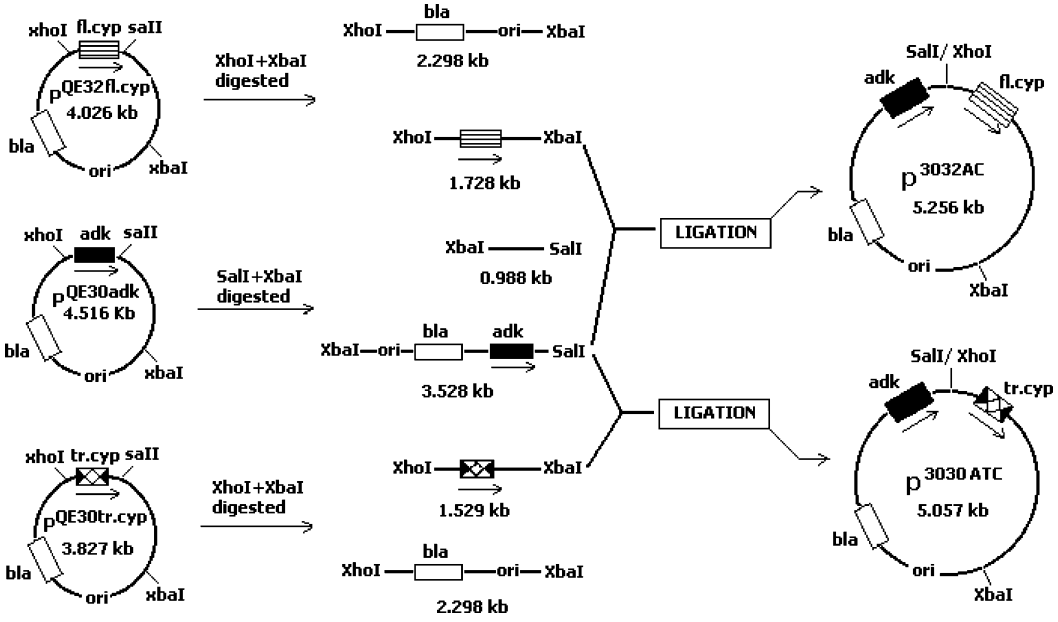

FIGURE 1: Schematic strategy for construction of bacterial coexpression vectors (p3032AC and p3030ATC) for simultaneous expression of AdK/LdCyP or AdK/N_{22-88}DEL LdCyP.

media, and induction was allowed to continue for 24 h. Cells were pelleted at the indicated time intervals and washed five times with 1× PBS to get rid of DSS present in the media, and samples were made by boiling the same amount of cells with SDS-containing sample buffer and analyzed as described above. The result will show that prior exposure of cells to DSS did not affect subsequent IPTG induction.

Construction of Coexpression Vectors. Three separate precursor plasmid constructs carrying expressible adenosine kinase (pQE30adk), full-length *ldcyp* (pQE32fl.cyp) and truncated (N_{22-88}DEL) *ldcyp* (pQE30tr.cyp) genes, respectively, were utilized for construction of two new coexpression vectors, p3032 adk + fl.cyp and p3030 adk + tr.cyp (denoted as p3032AC and p3030ATC, respectively, in the text) (Figure 1). Construction strategies of the precursor vectors have already been described in an earlier publication (*28*). The new coexpression vectors, p3032AC and p3030ATC, apart from having the common in-frame AdK-encoding sequence under the control of a T5 promoter, had either the full-length LdCyP (fl.cyp) or truncated LdCyP (tr.cyp) sequences inserted downstream of another T5 promoter. Details of the strategy are presented in Figure 1. Digestion of the plasmid pQE30adk with *Sal*I and *Xba*I produced two fragments of 0.988 and 3.528 kb sizes. The larger fragment contained the entire *adk* along with the complete upstream sequence necessary for transcription and translation. The ampicillin resistance gene and *oriC* sequence necessary for the replication of the plasmid were also retained undisturbed. The fragment (3.528 kb) after gel elution was ligated to either *Xho*I−*Xba*I digested 1.728 kb (in case of fl.cyp) or 1.53 kb (in case of tr.cyp) fragments isolated from either pQE32fl.cyp or pQE30tr.cyp, respectively. The resulting expression vectors, p3032AC and p3030ATC, thus constructed had all the characteristics of self-replicating plasmids besides having two separate T5 promoters and necessary transcription/translation recognition signals preceding two encoding sequences of interest. Since PCR was totally avoided during the construction, there was no possibility of change in the functional property of the protein products that often occurs during PCR amplification. Bacterial strain JM109 was transformed with the ligated plasmids and selected on LA-ampicillin plates. Transformed cells were further confirmed with cell-PCR using specific primers for AdK, fl.cyp, and tr.cyp. Plasmids were prepared from the positive clones and transformed into M15 cells for expression.

Coexpression Methodology. M15 cells harboring either plasmid p3032AC or p3030ATC were grown in LB medium up to ~0.8A_{595} and induced overnight in the presence of 1 mM IPTG. Extracts from the resuspended cell pellet were prepared by sonication in lysis buffer (1 mM DTT, 1 mM PMSF, and 1 mg/mL lysozyme). The clarified supernatant was taken as the crude extract. Often, induced cells were directly boiled in SDS buffer to check expression. Coexpression was confirmed by running the total cell extract or the centrifuged crude extract on 15% SDS−PAGE. Bands were visualized after staining with Coomassie blue or after immunoblotting with polyclonal antisera against both AdK and LdCyP.

AMP Formation in vivo. To measure the intracellular AMP formation, cells were grown at 37 °C up to an OD_{595} of 0.8 in LB medium. IPTG (1 mM) was added, and incubation was continued for further 2 h. Cells, withdrawn from the respective cultures, were harvested and washed in ice-cold PBS twice. The cell pellet obtained was suspended at a concentration of 10^7cells/mL in carbon-source depleted minimal medium as described elsewhere (*37*). To the cell suspension (100 μl), [^{3}H]-Ado (2000 cpm/pmol) was added to a final concentration of 4 μM, and incubation was carried out at room temperature. At indicated time intervals, samples were dipped into a boiling water bath and centrifuged after sonication. An aliquot (25 μl) was withdrawn from the clarified supernatant and assayed for the AMP formation after spotting on DE-81 paper (*32*).

RESULTS

Kinetics of AdK Refolding in the Presence and Absence of Cyclophilin. Exposure of proteins to denaturant results in

generation of molecules having various unfolded states, which are in equilibrium with each other (*38*−*40*). Therefore, it was necessary to obtain information about the ratio of each type of unfolded AdK structures formed under the condition of treatment. To determine this, AdK was denatured for 1 h in the presence of 6 M urea. Tryptophan fluorescence (λ_{exc} = 295 nm) emission spectra of the native, unfolded, and refolded enzyme revealed that the emission peak of the native protein was red-shifted upon denaturation with concomitant fluorescence quenching (Figure 2A). However, in refolding a significant amount of tertiary structure is regained within the mixing period (10 s) when compared to the fluorescence signal of the native protein. The nature of the spectra remained same even after 30 min, indicating no further refolding at the later stage (data not shown).

Having confirmed unfolding and rapid refolding of AdK, we set out to determine reactivation yield of the enzyme upon renaturation. The results in Figure 2B indicate that reactivation of the enzyme molecules, regardless of the presence or absence of chaperone, followed a similar biphasic pattern. Moreover, the extent of the refolding yields as measured by recovery of activity was same in both cases. Within the first minute, about 23−26% of the activity was regained rapidly with an apparent rate constant (k_1) of around 3×10^{-2} s^{-1} and $t_{1/2}$ of ~23 s.

Thereafter, the rate of reactivation slowed drastically with about 28−32% of the activity being recovered after 2 h. The kinetics of reactivation was therefore typical of fast-refolded conformers (U_F) as shown by Kern *et al.* (*38*). Experiments displayed in Figure 2C showed no improvement in the activity pattern even when refolding was carried out in the presence of increasing concentrations of LdCyP. Clearly, the remaining amount (~68−72%) of the denatured protein, which could not be reactivated even after 2 h, possibly formed irreversible aggregates (*39*). Taken together, these results are consistent with the interpretation that the PPIase activity of LdCyP is not responsible for facilitating the rate of conversion of the fast-refolded form of AdK (*38*). From these experiments, it became clear that under the present denaturation/renaturation conditions, neither PPIase nor chaperone activity of LdCyP is needed to facilitate reactivation of AdK.

LdCyP or Its Truncated Version Helps To Sustain AdK Activity. Since under the given condition of denaturation, a fraction of denatured AdK renatured spontaneously without the help of LdCyP or its truncated version, we took advantage of this condition and denatured soluble aggregates of AdK (5 μM) with 6 M urea. After complete denaturation, as determined by functional activity assay, the solution containing the enzyme−urea mixture was divided into four equal parts. Whereas one part was rapidly diluted by adding into assay mixture (*32*), the other three parts were similarly diluted in the mixture containing (i) LdCyP (4.5 μM), (ii) N_{22-88}DEL LdCyP (200 nM), or (iii) LdCyP (4.5 μM) and CsA to reduce the final concentrations of urea and AdK to 60 mM and 50 nM, respectively. Immediately after dilution to enzyme-assay reaction mixture, the kinetics of all the reactions was followed. As expected, results of such experiments indicated that the extent of AMP formation was similar and maintained linearity for the first 15 min in all combinations tested (Figure 3A). However, with the progress of time, the reaction mixture without the chaperone ceased to follow

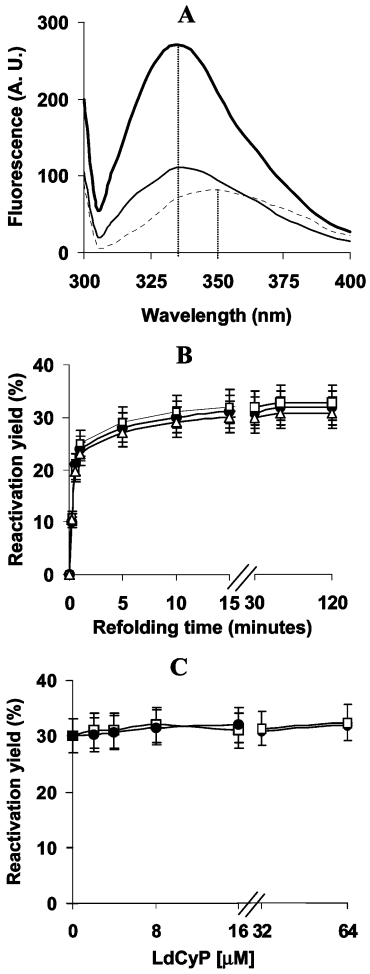

FIGURE 2: Panel A shows tryptophan fluorescence spectroscopy of native (—), denatured (− − −), and refolded (−) AdK. The change in fluorescence emission of the native protein (1 μM) was monitored either after denaturation or immediately after transfer of the denatured protein to refolding conditions. Panel B shows the effect of LdCyP or its truncated version on the reactivation yield of AdK. Urea-denatured enzyme (5 μM) was appropriately diluted in the reactivation buffer containing LdCyP (■), its truncated version (□), or no chaperone (△). At the indicated times, aliquots were quickly withdrawn and assayed for 2 min according to the procedure described in Materials and Methods. The plots represent the average of three experiments. In panel C, the recovery of the activity from the denatured enzyme refolded for 15 min (■) and 2 h (□) in the presence of increasing concentrations of LdCyP was monitored.

time-dependent linear kinetics and started to level off after 15 min, suggesting inactivation of the enzyme. In contrast, reaction mixtures that received (i) LdCyP, (ii) N_{22-88}DEL LdCyP, or (iii) LdCyP and CsA from the beginning continued to follow linear kinetics, indicating that the observed inhibition of the enzyme activity after 15 min was certainly not due to limitations in the assay condition. Presence of CsA did not have any inhibitory effect on the kinetics of the reaction. Since, the kinetic pattern displayed in all the reactions remained similar during the initial 15 min regardless of the presence or absence of chaperone and did not show hyperbolic or sigmoidal kinetics during the first 15 min, we concluded that chaperone activity of LdCyP did not have any function during the first 15 min but must be playing some essential function in a CsA-insensitive manner during the later part of the reaction.

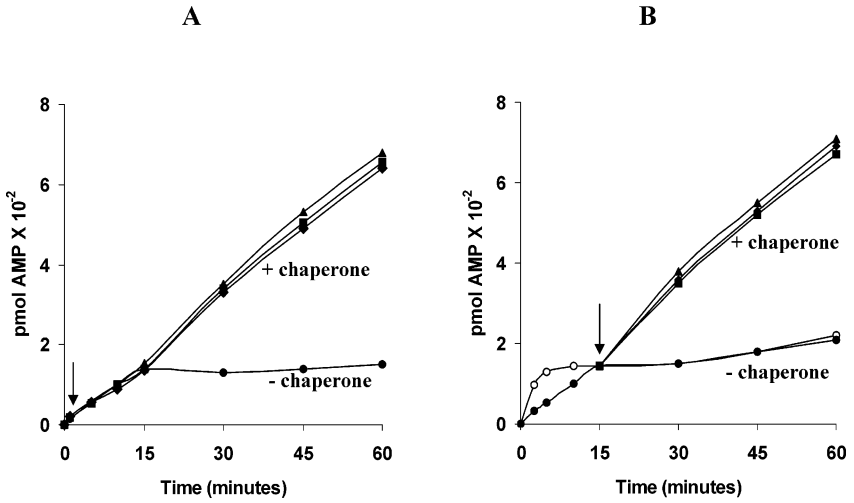

FIGURE 3: Effect of LdCyP or its truncated version during renaturation of AdK. Recovery of activity from the completely denatured protein was monitored under renaturing condition in the presence of LdCyP (■), its truncated version (▲), and LdCyP with CsA (▼). In panel A, the assay of urea-diluted enzyme was carried out in the presence of chaperone from the beginning, while in panel B chaperone was added after 15 min (⇓). A parallel reaction in the absence chaperone served as the control (●). The symbol (○) represents reaction kinetics of native AdK. For details, refer to Materials and Methods.

LdCyP and Its Truncated Version Prevent Aggregate Formation. The role of CyP in folding and assembly of many but not all proteins is well documented (*30*, *41*). To investigate in detail the step where LdCyP acts to keep AdK active over a long period of time in an isomerase-independent manner, a similar but not identical experiment as described in Figure 3A was designed. Since all the reactions with various combinations described above (Figure 3A) displayed linear kinetics up to 15 min either in the presence or in the absence of LdCyP, in the present experiment, the kinetic patterns of the urea-denatured enzyme were monitored after carrying out an identical requisite dilution with the assay mixture. At the 15th minute, the reaction mixture was split into four equal parts, and chaperone (as indicated) was added to three of the assay mixtures at that instant (Figure 3B). Parallel with these experiments, a control AdK reaction without LdCyP was also run with the same amount of native enzyme. As was seen in Figure 3A, all the reactions during the first 15 min followed similar linear kinetics. Results further indicated that the kinetic pattern of the native enzyme (control reaction), except the initial burst during the early stage of the reaction, followed a sluggish nature similar to the renaturing reaction mixture that did not receive any chaperone (Figure 3A,B). In contrast, reaction mixtures that received LdCyP or its truncated version at the 15th minute continued to follow linear kinetics (Figure 3B). CsA, again, did not exert any inhibitory effect. From these results, it became clear that the reduced rate of AMP synthesis by the refolding enzyme as compared to the native (control) enzyme during the initial 15 min was due to the average rate displayed by the mixture of folded and unfolded enzyme molecules (Figure 3A,B). Furthermore, since the tendency of the enzyme to get inactivated could be prevented or overcome by LdCyP or its truncated version even in the presence of CsA, the preceding results together with our previous observations (*28*) are consistent with the interpretation that the chaperone function of LdCyP must be involved in prevention of aggregation and disaggregation of the protein, thereby ruling out the possibility of involvement of isomerase activity at any stage of the process.

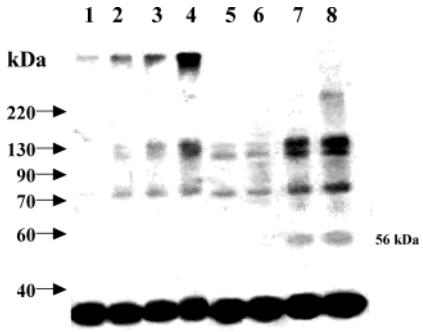

FIGURE 4: DSS-mediated protein cross-linking experiment to monitor aggregate formation during incubation under renaturation conditions. The extent of AdK aggregate formed during renaturation was followed in the absence (lanes 1−4) and in the presence (lanes 5−8) of LdCyP. During incubation, samples were withdrawn at 5, 15, 30, and 60 min (left to right) and analyzed (for details, see Materials and Methods).

Aggregate-Trapping Experiment. In our previous studies, we presented evidence to show that LdCyP could rescue functional monomers from already formed AdK aggregates (*28*). By developing an aggregate-trapping strategy, here it is directly shown that LdCyP prevents aggregation after refolding as well. For this, completely denatured enzyme (10 μM) was allowed to renature (1 μM) in the presence of either homobifunctional cross-linker DSS alone or DSS along with LdCyP. At various time intervals (5, 15, 30, and 60 min), aliquots (2 μg of AdK) from the reaction mixtures were withdrawn, run on 10% SDS−PAGE, and immunoblotted with anti-AdK antisera. Results clearly indicated that during the incubation period, AdK formed large aggregates along with lower (di-, tri-, and tetra-) oligomers (Figure 4). With increase in the time of incubation, there was increase in the formation of larger aggregates found at the top of the gel (lanes 1−4). Interestingly however, when LdCyP was present during the incubation (lanes 5−8), the higher molecular weight aggregates could not at all be detected. Instead, the formation of lower oligomers was clearly visible along with appearance of the ~56 kDa band corresponding to the AdK−LdCyP complex (*28*). From these experiments, it was

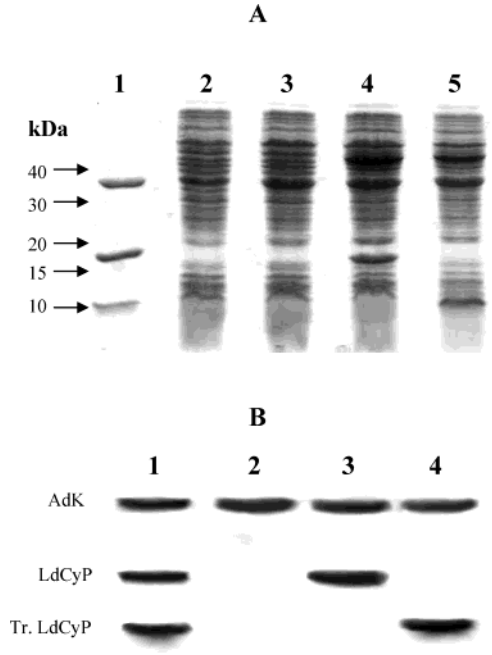

FIGURE 5: Coexpression (A) and characterization (B) of both AdK and LdCyP or both AdK and N_{22-88}DEL LdCyP in *E. coli* M15. In panel A, crude extracts prepared from IPTG-induced *E. coli* M15 cells carrying AdK (lane 3), AdK and LdCyP (lane 4), and AdK and N_{22-88}DEL LdCyP (lane 5) encoding sequences were run on 15% polyacrylamide gel and analyzed after Coomassie staining. Lane 1 represents purified AdK, LdCyP, and N_{22-88}DEL LdCyP used as marker, whereas lane 2 received crude extract prepared from control M15 cells. Arrows at the extreme left indicate corresponding positions of prestained molecular weight markers. In panel B, proteins from lanes 1 and 3−5 of the gel in panel A were transferred to nitrocellulose paper and immunoblotted with AdK and LdCyP antibodies (lanes 1−4).

abundantly clear that (i) formation of higher order aggregates indeed caused inhibition of catalytic activity of AdK and (ii) LdCyP plays a crucial role of preventing formation of these aggregates, besides removing them.

Characterization of the Coexpression Vectors. From all our preceding experiments and other published results, it was evident that the aggregation of proteins under various conditions is a problem of general interest and therefore is physiologically relevant (*28*, *42*−*44*). In order to have more direct evidence in favor of this possibility, we designed and constructed two coexpression vectors (p3032AC and p3030ATC) for simultaneous expression of both AdK and LdCyP or AdK and N_{22-88}DEL LdCyP, respectively, in *E. coli* M15 (Figure 1, Materials and Methods). The M15 cell clone harboring only pQE30 + AdK was also used as control. Results of the coexpression experiment are presented in Figure 5. The polyacrylamide gel electrophoresis pattern of IPTG-induced crude extracts from the respective plasmid-carrying cells (Figure 5A) and the corresponding immunoblot experiments (Figure 5B) using antibody mixture containing polyclonal antibodies of both AdK and LdCyP clearly demonstrated simultaneous expression of both the proteins in almost equimolar proportion.

Cells Coexpressing Both Proteins Show Higher AdK Activity Compared with Cells Expressing AdK Alone. Having been successful in constructing cell clones capable of expressing both the proteins simultaneously, we set out to analyze the activity of AdK and its status *in vivo* in terms

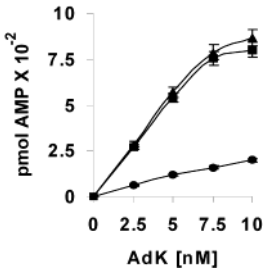

FIGURE 6: Crude extract from cells coexpressing AdK and LdCyP (■) or AdK and N_{22-88}-DEL LdCyP (▲) displays higher specific activity than crude extract prepared from cells expressing AdK alone (●).

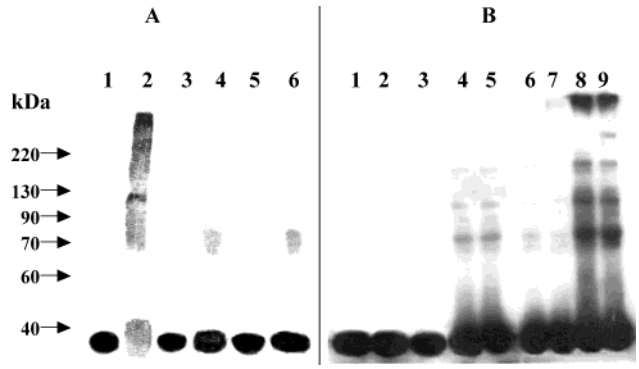

FIGURE 7: Analysis of AdK cross-linking in the coexpressed cells: (A) extracts containing 5 μg of AdK prepared from cells expressing AdK alone (lanes 1 and 2), AdK and LdCyP (lanes 3 and 4), or AdK and N_{22-88}DEL LdCyP (lanes 5 and 6) were treated with (lanes 2, 4, and 6) and without (lanes 1, 3, and 5) DSS, and (B) extract was prepared from the same number of DSS-exposed (lanes 4−9) and unexposed (lanes 1−3) cells expressing AdK alone (lanes 3, 8, and 9), AdK and LdCyP (lanes 1, 4, and 5), or AdK and N_{22-88}DEL LdCyP (lanes 2, 6, and 7). From both set of experiments, equal amounts of extract were run on 10% SDS−PAGE. The protein from the gel after transferring to nitrocellulose was immunoblotted using AdK antibodies. For details, refer to Materials and Methods.

of aggregation. To carry out this experiment, equivalent amounts of AdK protein, as determined by densitometric scanning of Western blot of crude extracts, from three separate clonal populations of cells expressing AdK with or without LdCyP and N_{22-88}DEL LdCyP were assayed for AdK activity. Results clearly demonstrated several fold higher levels of specific activity in extracts of cells that coexpressed LdCyP or N_{22-88}DEL LdCyP along with AdK in comparison with the extract made from cells expressing AdK alone (Figure 6). This is consistent with all our earlier observations showing activation of AdK by LdCyP or its truncated version.

AdK Does Not Aggregate in vivo When Coexpressed Along with LdCyP or with Its Truncated Version. With the use of the purified system, our earlier results had demonstrated that disaggregation of AdK by LdCyP resulted in activation of the enzyme (*28*). To examine whether the activation observed above was due to a similar phenomenon occurring *in vivo*, we carried out cross-linking of AdK both *in vitro* and under simulated *in vivo* conditions using the newly constructed cell clones. In the first set of experiments (Figure 7A), crude extracts from cells expressing (i) AdK, (ii) AdK and LdCyP, and (iii) AdK and N_{22-88}DEL LdCyP were subjected to cross-linking with DSS (0.5 mM). Analysis of an equivalent amount of DSS-exposed AdK from each reaction mixture

on SDS−PAGE clearly demonstrated formation of the high molecular weight cross-linked AdK aggregates in crude extract expressing only AdK (lane 2). In contrast, a very insignificant amount of cross-linked product was noticed in extracts of cells where coexpression of AdK occurred along with LdCyP or N_{22-88}DEL LdCyP (lanes 4 and 6, respectively). Lanes 1, 3, and 5 were control experiments run without DSS. The most notable aspect of this experiment was the near quantitative conversion of AdK as monomer in extracts containing LdCyP or N_{22-88}DEL LdCyP (lanes 4 and 6).

To rule out that the observed disaggregation did not occur during and/or after preparation of the crude extract, intracellular protein cross-linking was performed (Figure 7B). In this experiment, grown cells (OD_{595} = 0.80) were exposed to DSS (0.5 mM) prior to IPTG induction (lanes 4−9). At different post-induction times, extracts made from the washed cells were analyzed. Results indicated that despite facile expression of AdK, cells coexpressing both the proteins hardly formed any large cross-linked aggregates even after 24 h postinduction (lanes 4−7). In contrast, large cross-linked aggregates were clearly visible on the top of the gel when extract made from the cells expressing only AdK was loaded (lane 8 and 9). The only difference that we observed here from our *in vitro* cross-linking experiment (Figure 4) was the absence of AdK−LdCyP cross-linked product (∼56 kDa band). We attribute this to be due to the transient nature of interaction that could not be controlled under *in vivo* condition. Taken together, these results are consistent with the interpretation that (i) LdCyP prevents aggregate formation *in vivo* as well and (ii) the higher activity of AdK observed in the crude extract of cells coexpressing both the proteins was clearly due to availability of AdK in the monomeric state, thus supporting our *in vitro* results.

Facilitated AMP Synthesis in E. coli Coexpressing Both AdK and LdCyP Compared with That in Cells Expressing AdK Alone. It is well established that *E. coli* cells are deficient in AdK (*45*). Moreover, taking advantage of the interlocked coupling between transport and metabolism, various laboratories have shown that AdK is one of the enzymes that, due to its Ado phosphorylating ability, provides the driving force for the movement of Ado from the extracellular environment into the cell (*46*−*48*). This mechanism enables cells to maintain the intracellular concentration of adenosine nucleotide. Since the tendency of AdK aggregation could be minimized in cells coexpressing both the proteins, we utilized this observation to investigate intracellular AMP formation in our constructed cell clones. Results show that as compared to pQE transformed control cells the extent of AMP formation was higher in cells expressing AdK alone (Figure 8). Interestingly, the rate of AMP synthesis was many fold higher in cells that coexpressed LdCyP or its truncated version along with AdK. Since, all of our earlier studies demonstrated that AdK is more active as monomer and not so active in its aggregated form, the results favor the interpretation that increased formation of AMP in the cells coexpressing both the proteins was clearly due to availability of more AdK in the monomeric state.

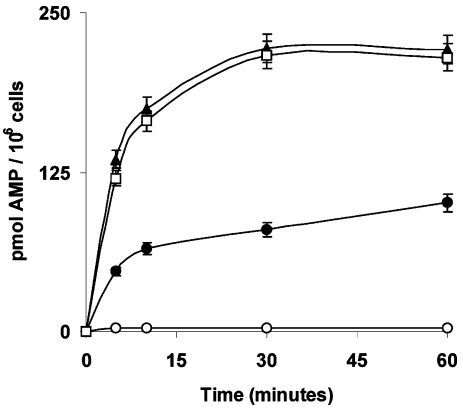

FIGURE 8: Kinetics of AMP formation in cells expressing AdK alone (●), AdK and LdCyP (▲), AdK and N_{22-88}DEL LdCyP (□), and control M15 (○). Experimental details are in the Materials and Methods section.

DISCUSSION

Based on extensive studies, the PPIase activity of CyPs, along with protein disulfide isomerases (PDIs) and other similar classes of proteins, has been designated as "folding catalysts" rather than "chaperones" (*30*). Approaches made by various groups of workers suggested that CsA-sensitive PPIase activity of CyPs functions during refolding of *in vitro* denatured proteins (*38*, *49*−*51*). Our recent observation on the "isomerase-independent" chaperone function of a single-domain CyP has certainly provided an added dimension in this area of research (*28*). In this connection, it must be mentioned that a similar type of activity displayed by FKBP 73 from wheat has recently been reported (*52*).

The novelty and uniqueness of our finding rests on the use of the native protein substrate (AdK) that forms soluble aggregates (oligomers) under natural conditions as opposed to stressed conditions such as heat or chemical shocks. To determine whether LdCyP or its truncated version play any role in refolding of the protein, we completely unfolded the enzyme with conventional method and subsequently followed recovery of the enzyme activity under refolding condition using both discontinuous and continuous assay methods (*53*). Our results indicated that, both with and without LdCyP or its truncated version, only 23−26% of the input AdK (50 nM) was productively folded within a very short period (60 s), after which not much appreciable refolding could be observed. Whether this was due to diversion of the major portion of the unfolded proteins permanently towards off pathway will however require future detailed studies using activity-independent parameters. In this connection, it must be mentioned that the formation of irreversible aggregates during refolding of rhodanese has already been demonstrated (*39*). Consequently, the permanently buried non-native prolyl-peptide bonds present in these aggregates could also be an additional reason for nonobservance of a slow-folding step and hence the lack of effect of LdCyP, an inherent limitation of *in vitro* refolding. In any case, the lack of effect of LdCyP on the refolding kinetics of AdK implied that, under the present condition, the denatured enzyme, in order to fold, did not require chaperone help (Figures 2 and 3). This observation is thus similar to a recent report that suggests nonessentiality of trigger factor PPIase in folding of cytosolic proteins of *E. coli* (*54*). However, the chaperone had definite influence at a later stage in sustaining the activity of the enzyme. A direct "aggregate trapping experiment" clearly showed that LdCyP indeed prevents AdK from aggregating (Figure 4). Taken together, these results provide

a direct *in vitro* demonstration that the CsA-insensitive chaperone function of LdCyP operates at the very step that prevents AdK from aggregating during or after productive refolding.

To investigate whether LdCyP or its N-terminal truncated version, like *in vitro*, also prevents aggregate formation under *in vivo* conditions, we designed vectors that simultaneously expressed both AdK and LdCyP or its truncated version in *E. coli*. Several workers in the past have used a similar coexpression strategy to study protein−protein interactions *in vivo* (*55*−*60*). However, the strategy that we adapted had several distinct advantages over others. It consisted of the following unique features: (i) the presence of two identical promoters in the resulting plasmid allowed the induction from the two different encoding sequences to take place in almost 1:1 proportion; (ii) the size of the coexpression plasmid was well within the acceptable range that could easily be transformed; (iii) unlike *L. donovani*, *E. coli* is known to be deficient in endogenous AdK. Therefore, AMP formation would be expected to be largely expedited by the availability of the inducible active intracellular AdK.

When both the proteins were expressed *in situ* using such a coexpression vector, it became abundantly clear that LdCyP or its N-terminal-deleted truncated version had a distinct influence on *in vivo* AdK both its aggregation status and in terms of its functional property as well. Protein cross-linking experiments and functional activity analysis carried out *in vivo* and *in vitro* with cells coexpressing both AdK and LdCyP or its truncated version undoubtedly established that higher specific activity of AdK resulted due to nonaggregation of AdK in these cells (Figures 6 and 7). Furthermore, the increased rate of conversion of Ado to AMP in cells coexpressing both AdK and LdCyP or its truncated form as compared to cells producing AdK alone (Figure 8) provided additional support in favor of the interpretation that LdCyP must have assisted AdK to remain as monomer in the coexpressed cells. These results therefore, together with our previous studies, are consistent with the explanation that the chaperone function (both pre- and post aggregation) of LdCyP can be functional both *in vitro* and under *in vivo* conditions. In support of this, two compelling evidences are in place. From the studies on the aggregate formation, carried out *in vitro* with various concentrations of the enzyme (extending from physiologically suboptimal to optimal) and simulated *in vivo* conditions, it appears that oligomerization is an inherent property of this protein and therefore is not conditional. Secondly, we consistently observed that the crude extract of IPTG-induced cells displayed higher enzyme specific activity in comparison to its highly purified state (data not presented). Since *E. coli* cells are enriched with GroEL and other chaperonic proteins that are also known to disaggregate and reactivate aggregated proteins to some extent, it is presumed that the higher specific activity observed in the crude extract was partly due to the chaperone function of these proteins (*61*). Our studies demonstrating further stimulation of AdK activity in the cells coexpressing both AdK and LdCyP or its truncated version reaffirmed that the endogenous bacterial chaperones were indeed not sufficient to disaggregate and reactivate proteins completely.

The endoplasmic reticulum (ER) is known to harbor a large heterologous chaperone−protein complex, comprising BiP, GRP94, calreticulin, and other ER-resident proteins including CyP, believed to be responsible for inhibiting premature misfolding or aggregation of nascent polypeptides or both (*62*−*64*). In view of such a large redundancy of multiple chaperonic proteins, it still remains to be known whether the disaggregating property of ER-located LdCyP observed here operates in tandem with all other chaperonic proteins or acts as an independent entity. Another possibility, as has been suggested earlier (*65*), would be that aggregation-prone ER proteins, during their refolding, might first bind to LdCyP and then "roll over" to another chaperone as per their requirements. Although it has not yet been possible for us to test the disaggregating property of LdCyP using any aggregation-prone membrane protein as substrate, the fact that cytoplasmic peptidyl-prolyl isomerase, either alone or in combination with DnaK, expresses its chaperone function in the cytoplasm of organisms is well-known (*66*, *67*). Given this scenario, it is conceivable that the disaggregating property of LdCyP demonstrated here would be functional in maintaining aggregation-prone ER-associated proteins in the monomeric state as well. The facts that (i) peptidyl-prolyl isomerases are known to bind several nascent ER-proteins, (ii) regulation of protein secretion through ER is dependent upon controlled aggregation−disaggregation of proteins, (iii) ER chaperones are known to be involved in maturation of several ER-resident proteins, and finally (iv) AdK activity in the coexpressing cells is much higher in comparison to cells expressing AdK alone support such a paradigm (*68*−*70* and this paper). Therefore, taking into consideration all the options discussed above, we present a composite schematic model highlighting the mechanistic steps that might be involved in disaggregation and reactivation of AdK by LdCyP (Figure 9). In the predicted model, we envisage that in the coexpressing *E. coli* cells, LdCyP, either alone or in conjunction with other *E. coli* cytosolic chaperone proteins, homologues of which and commonality of their functions have been found both in cytosol and in ER of other eukaryotes and *Leishmania* species as well, carries out disaggregation as well as prevention of aggregation functions, leading to activation of the enzyme.

Protein aggregation, often, is known to cause deleterious effects both on cell survival and in industrial front as well. Work from various laboratories has established that aggregated proteins, formed upon exposure of cells to either heat or high concentration of alcohol, can be disaggregated and reactivated by a number of heat-shock chaperones (*29*, *67*, *68*). However, despite these evidences, there are only a few straightforward experimental data to show that the chaperone function of a given protein resulted in regeneration of activity of the target protein on which the chaperone has actually worked. Our fortuitous discovery, showing that the natural aggregates of *L. donovani* AdK can be disaggregated and reactivated by a CyP from the same source, is consistent with an earlier report that demonstrated CyP-dependent stimulation of functional activity of an enzyme (*69*). Since secretion of proteins is also known to be dependent on their aggregation−disaggregation status (*64*), it appears that the ATP-independent "chaperone function" of the ER-located LdCyP demonstrated here may have important intracellular functions and various other ramifications in terms of secretion of ER-located aggregation-prone proteins and their maturation in general. Although, with regard to *Leishmania* physiology, we do not have any direct evidence yet to show

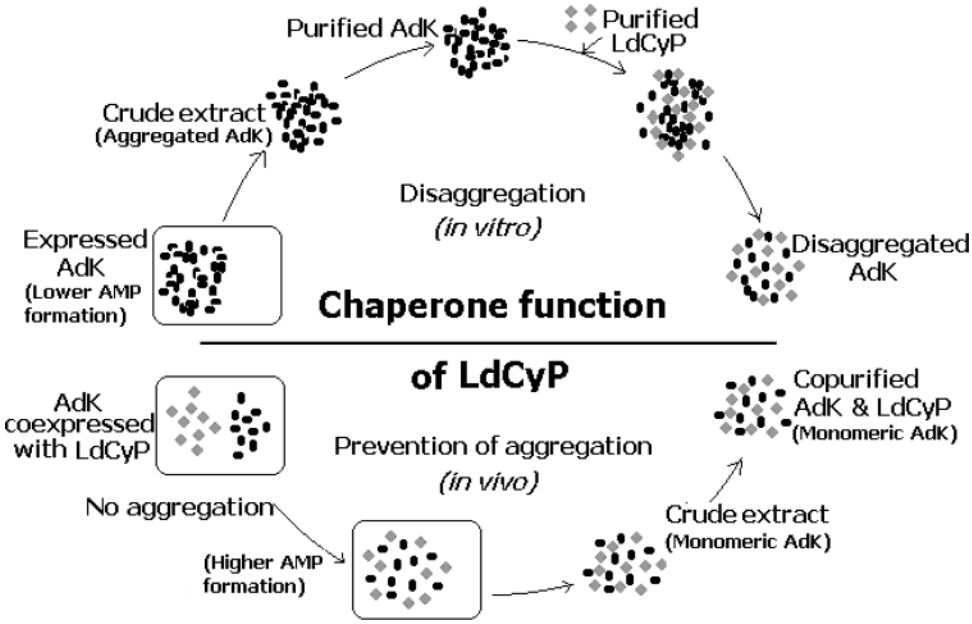

FIGURE 9: Composite schematic model showing chaperone function of LdCyP both *in vitro* and *in vivo*.

that the phenomena described above indeed operate in the parasite, the results obtained with *E. coli* under simulated *in vivo*-like conditions are noteworthy. In this connection, it is also to be pointed out that during morphological transformation of various parasites, the levels of a large number of enzymes, proteins, or both including various chaperones and cyclophilins are stage-specifically altered (*24*−*26*, *70*). Therefore, the possible role of LdCyP in maintaining ER-associated aggregation-prone proteins in an activated state also cannot be ruled out. Nevertheless, our ability in developing a system to disaggregate protein aggregates in *E. coli* underscores the possibility of exploring this strategy in biotechnological industrial fronts where aggregation of proteins, even under conditions favoring the native state, and their consequent inability to secrete out of the cells often create major problems (*71*).

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