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Protein-protein interaction plays a major role in regulating various biological processes. There is support for the view that CyP-like proteins may assist in the folding of proteins (327). Evidence also exists to indicate that PPIase may play a role in facilitating protein-protein interaction required for assembly (or disassembly) of multiprotein complexes (301). Our observation on the role of LdCyP in reactivation of inactive AdK aggregates by an isomerase-independent manner led to a host of interesting possibilities. So far, it was known that LdCyP interacted with AdK while carrying out the reactivation process. It also appeared that disaggregation of the AdK aggregates was indeed responsible for the dramatic stimulation of the parasitic enzyme. However, based on the previous data, mechanistic approach for detailed understanding of the CyP-mediated reactivation process and its relevance with the disaggregation was ill-understood. Additionally, in terms of reactivating property, the individual effect of LdCyP on two different types of aggregates of AdK, namely natural aggregate and ADP-induced aggregate, remained completely unknown.

It was apprehended from the results presented in the preceding chapter that natural aggregate of AdK was resistant to LdCyP-mediated disaggregation process. This observation motivated us to determine the relationship between the disaggregation and reactivation of AdK. Mechanistic details of how LdCyP reactivates exclusively the ADP-induced aggregates are presented in this chapter. Unstable ADP-induced AdK-aggregates, which were disaggregated by LdCyP, eventually participated further in reactivation. The activity revived with decrease in the ADP-induced aggregate size. Result also indicated that displacement of ADP from the ADP-aggregated enzyme by LdCyP resulted in reactivation. Since the importance of AdK in Ado homeostasis and in maintenance of intracellular AMP/ADP level is crucial especially for purine auxotrophs, implication of these results in the context of modulation of activity of the enzyme through interplay of ADP and/or LdCyP interaction assumes importance during morphological transformation of the parasite. Furthermore, the information generated in this study highlighted the process by which LdCyP exerts its unique chaperonic function to disaggregate and reanimate the AdK-enzyme substrate.
3.1. "Natural Aggregate" of AdK is not reactivated by chaperone:

Purified AdK enzyme comprised of mixture containing monomers and oligomers. Since AdK has the natural tendency to aggregate by itself and form 'natural aggregate', we were interested to separate monomers from the natural aggregate by gel filtration chromatography of the purified enzyme. To track the phosphorylating efficiency of the HPLC-isolated monomeric form of the enzyme, its activity was monitored in presence of either ADP or LdCyP and in presence of combination of both (Table 5).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LdAdk Activity (nmol x 10⁻²/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Natural Aggregate</td>
</tr>
<tr>
<td>AdK</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>AdK + CyP</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>AdK + ADP</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>AdK + ADP + CyP</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

Table - 5: Activity of monomeric and aggregated form of AdK in presence of LdCyP and ADP. Activity of monomeric and aggregated forms were determined in presence of either LdCyP or ADP and in presence of combination of both. AdK concentration was 50 nM. LdCyP and ADP concentrations were 20 and 400 μM respectively. AMP produced after 30 min of each reaction is presented. Due to continuous generation of endogenous ADP, the AdK (monomer) assay represents inhibited activity. Hence, for comparison, AdK (monomer) assay in presence of LdCyP that followed time-dependent linearity was taken as 100%.

Results showed that the monomeric enzyme was inhibited by exogenously added ADP. AdK (monomer) displayed higher activity in presence of LdCyP. Assuming such stimulated activity as 100%, it was seen that extraneous ADP inhibited the activity by about 83%. This inhibited activity was again stimulated upon inclusion of LdCyP and attained a level (93%), close to the activity observed in presence of LdCyP. This result clearly indicated that although the amount of endogenously produced ADP was much low in comparison to exogenously added ADP, the enzyme inhibition (72%) without extraneous ADP was highly efficient. This very stimulation was attributed to fact that ADP produced during the 30 min reaction time had its share of inhibitory effect. In
contrast, the low level of activity, displayed by the aggregated enzyme, remained more or less unaffected in presence of either ADP or LdCyP.

3.2. Disruption of the ADP-induced AdK aggregates displayed increased activity:

Earlier it was reported that of the two reaction products, AMP and ADP, accumulation of ADP but not AMP of the adenosine phosphorylation reaction facilitated formation of the AdK aggregates, resulting in inhibition of the reaction. Result of table 5 confirmed ADP-dependent inhibition theory. Result of table 5 also helps to comprehend that two types of aggregates are formed by LdAdK which is dependent on the presence of product ADP. One is 'natural aggregate', and another is ADP-induced aggregates. To investigate whether both forms of aggregates were consumed by LdCyP-chaperone as protein-substrate, chaperone-mediated reactivation kinetics of the purified enzyme, comprising of both naturally aggregated and monomeric forms of the enzyme, was followed under two conditions (Fig. 3.1). In the first set of protocol, prior to initiation of activity analysis of the enzyme with [3H]-Ado, the purified enzyme was preincubated with LdCyP for 15 min whereas in the second set of reaction, the preincubation of the enzyme with LdCyP was omitted and the phosphorylation reaction was set off with addition of [3H]-Ado and chaperone simultaneously. It was reasoned that if the natural aggregates also participated as substrate, the chaperone-preincubated sample, in principle, should display higher rate of reaction due to participation of larger number of AdK molecules at saturating [3H]-Ado concentration. Interestingly, the results showed that under either condition the rate of the reaction as evident from the AMP yield remained identical all through, thereby indicating that the natural aggregates were not utilized as substrate by LdCyP. The results of Table 5 and figure clearly indicate that chaperone reactivates only those oligomers that are formed due to continuous production and/or addition of exogenous ADP during the course of the enzymatic reaction.
3.3. LdCyP displaces ADP from the ADP-bound AdK aggregate:

**Figure 3.2**: Reactivation following removal of ADP from the ADP-bound AdK aggregate. The ADP-aggregated enzyme, eluted from the gel-filtration column, was separately pretreated with LdCyP (■), PK+PEP (□), PEP (●), PK (○) and without treatment (0), was used in enzyme activity assays.

**Figure 3.3**: PK-LDH coupled NADH-oxidation assay, using ADP-aggregated enzyme as the source of "substrate ADP", was performed in presence (■, ▼) and absence (●, ◆) of LdCyP (1 μM). The aliquots of ADP-aggregate used in assays (●, ■) and (◆, ▼) was 50 and 100 μl respectively. The symbol (▲) shows isolate natural aggregate assay in presence of LdCy (10μM). NADH oxidation was monitored by dro in $A_{340nm}$.

Overall strategy of the experiments presented in this section is based on the premise that since ADP caused aggregation and inactivation of the enzyme, the ADP-bound enzyme-aggregate should be weakly active as long as ADP was bound to it. Given this, removal of ADP from the aggregated enzyme should reactivate the enzyme. ADP-aggregated enzyme was isolated from the void peak of the gel filtration column. Herein, evidence is presented to show that pretreatment of the aggregate with either (i) PK along with PEP, or (ii) LdCyP-chaperone led to reactivation of the enzyme. The treated ADP-aggregated enzyme, as opposed to the untreated enzyme, displayed enhanced reaction rate as evident from the increased [3H]-AMP yield (Fig. 3.2). The higher rate was evident from the beginning of the reaction. The reaction-rate of the PEP/PK-treated enzyme was almost comparable to the rate at which LdCyP-exposed enzyme functioned. However, PK or PEP alone was incapable of reactivating the enzyme.

In the experiment described in Fig. 3.3, PK-LDH coupled assay was utilized to determine the ADP content in the ADP-enzyme aggregate and to demonstrate the influence of LdCyP on the release of ADP from the aggregated substrate. Results showed that, in comparison to the reaction without
LdCyP, there was a clear enhancement of the reaction rate in presence of LdCyP as measured by drop in $A_{340}$. With increase in ADP-enzyme concentration there was proportional increase in both reaction rate and the extent of ADP release that eventually culminated with "leveling off" of the reaction. Since progress of the PK-LDH coupled reaction is strictly dependent on the availability of free ADP, the most likely interpretation of increased rate of NADH oxidation in presence of saturating concentration of LdCyP must be due to LdCyP-facilitated rapid release of the bound ADP from the enzyme-aggregate. Calculations indicated that average binding stoichiometry between enzyme:ADP was 1:18. Overcrowding of the hydrophobic pocket of the enzyme active site by ADP, occurring due to aggregation-mediated steric modulation, possibly resulted in such a high stoichiometry. In this respect, the natural aggregate was inactive as substrate, indicating absence of ADP. It is possible that PK, due to its large molecular size, cannot as such get easy access to the ADP, caged within the hydrophobic pocket and/or interstitial space of the enzyme-aggregate. LdCyP, by interacting with the enzyme-aggregate, disrupts the structure leading to release of the ADP and allows the PK-LDH coupled reaction to proceed at a faster rate. Hence we conclude that whereas LdCyP acts by dispersing the enzyme-aggregate leading to release of ADP, PK-PEP combination indirectly does the same job by converting enzyme-bound ADP to ATP, thereby apparently achieving the same end result i.e. enzyme reactivation. Exogenous ADP-induced aggregate, when used as substrate, behaved similarly (data not shown). The fact that LdCyP does not bind ADP directly, but increases the overall apparent $K_d$ of binding of the aggregated-enzyme for ADP by interacting transiently with it, is consistent with such an explanation.

3.4. Enzyme activity follows inverse relationship with the aggregate size:

From many experimental evidences, it is apparent that disaggregation of AdK by LdCyP leads to reactivation of the enzyme. With a specific experiment we now directly demonstrate that the enzyme activity is inversely proportional to the aggregation status of the enzyme. To provide such a proof, we, in absence and in presence of increasing concentrations of either form of the chaperone, measured the hydrodynamic diameters of AdK (Fig. 3.4) and plotted the highest peak diameters of the aggregated enzyme population, taken at the indicated concentrations of chaperones. In

![Figure 3.4: Activity of AdK is inversely proportional to its aggregate size formed in presence of ADP (100 μM). Decreasing mean hydrodynamic diameters obtained from DLS analysis of AdK (2μM) in the presence of increasing concentrations of either LdCyP (○) or Ndel CyP (•) were plotted against the corresponding AdK activity, measured in presence of indicated concentrations of LdCyP (□) and Ndel CyP (●).](image-url)
parallel, the specific activities of the enzyme at the indicated concentrations of the chaperones were also monitored. Results clearly show that with increasing concentration of either form of the chaperone, the hydrodynamic diameter of the aggregated AdK decreased gradually. Concomitant with such reduction in the hydrodynamic diameter, there was gradual increase in the specific activity of the enzyme that eventually reached a plateau (Fig. 3.4). From these results an inverse relationship between the activity of the enzyme and the status of the enzyme aggregation is being invoked.

3.5. Peptide sequence from residue 147-156 of CyP is responsible for reactivation of LdAdK:

Protein-protein interactions are generally mediated by binding of a short stretch of peptide from one protein to a large globular segment in another. Recent evidence have provided examples of hundreds new peptides binding to proteins. Moreover, some useful recent studies have revealed that peptide may act effectively as chaperone to disaggregate stable protease-resistant amyloid aggregates (328-331). It is also being claimed from these studies that short-length peptide chaperones may even inhibit the initiation of several neuro degenerative diseases.

In view of these observations and taking into account our results on the LdAdK stimulatory activity of a truncated CyP (N-del CyP), it was thought rational to make an effort to develop a peptide fragment of LdCyP that would be having the LdAdK reactivating activity. With this plan in mind, scanning of several different short peptide sequences from LdCyP was carried out. Interestingly, a small decapeptide from amino acid residue 147 to 156 appeared to have the LdAdK stimulating activity. Several experimental results are presented to examine the novel reactivation property of this peptide.
Due to difficulty in expression and purification of smaller fragments of LdCyP, we were compelled to use synthetic peptide fragments containing different regions of LdCyP. Initial designing of six different peptides, having different regions of LdCyP, failed to show reactivation of AdK. Finally, we were able to find out a decapeptide sequence, residue 147 to 156 (PeP-CyP^-iss), that was capable of stimulating the activity of LdAdK.

**Figure 3.5**: PeP-CyP$_{147-156}$ fragment inside the full-length amino acid sequence of LdCyP. Decapeptide region is shaded with gray color.
3.5.1. **PeP-CyP\textsubscript{147-156} efficiently reactivates inactived AdK:**

Having confirmed that full-length cyclophilin (LdCyP) disaggregated and reactivated the aggregated AdK of *L. donovani*, we were interested to find out the shortest part of the LdCyP that would be equally capable of exhibiting the similar AdK reactivating property. For this purpose, the extent of stimulation of purified LdAdK as a function of chaperone peptide and LdCyP concentration was first monitored. Two separate sets of Ado phosphorylation reactions of AdK were designed and after 30 minute of the reaction time, AMP yield of each experimental set was separately measured using standard radiochemical assay procedure (Fig. 3.6). In both reaction sets, specific activity of AdK was determined in presence of increasing concentrations of LdCyP (■) and PeP-CyP (♦), respectively. As a function of full-length LdCyP and/or peptide concentration the activity of purified AdK was plotted. This comparative assay experiment, for the first time revealed that, PeP-CyP\textsubscript{147-156}, which is a decapeptide fragment of LdCyP, reactivated purified AdK dramatically. More interestingly, this small part of the cyclophilin is almost equally efficient as the full-length LdCyP in reactivating AdK.

In order to assess the decapeptide-mediated reactivation pattern of AdK, the effect of adding PeP-CyP\textsubscript{147-156} at a time (40th min) when the progress of Ado phosphorylation reaction showed the signs of slowing down was followed (Fig. 3.7). Two separate sets of phosphorylation reactions in the absence and in the presence of 40 μM PeP-CyP\textsubscript{147-156} were started simultaneously using a fixed amount (50 nM) of AdK in each reaction. The control reaction, without Pep-CyP, was allowed to
proceed uninterruptedly for 120 minutes (Fig. 3.7), whereas in the second set, after a period of 40 minutes, 40 μM Pep-CyP was added in the reaction mixture and was monitored for 120 minutes. At the indicated time intervals, samples were withdrawn and processed for AMP formation. After the initial few minutes, the reaction without Pep-CyP, as expected, became sluggish.

In contrast, the second set of reaction, where chaperone peptide was added after 40 minutes time gap, progressed with higher reaction rate with increased yield of AMP. Like the full-length cyclophilin, the peptide-augmented stimulation of activity was instantaneous and significant.

3.5.2. PeP-CyP147-156 withdraws the inhibitory effect of ADP on AdK:

It is already established from the preceding data that ADP, one of the reaction products of the AdK-reaction, induced aggregation and leading to inactivation of the enzyme. When added exogenously with increasing concentration, ADP itself showed its inhibitory effect on the enzyme. Results presented in Chapter 1 clearly showed that both LdCyP and N-del CyP were able to reverse the inhibitory effect of ADP. In order to assess whether the Pep-CyP exerted any withdrawal effect on the ADP-mediated inhibition, the experiment was carried out with two different sets of reaction. In the first reaction set, exogenous ADP was added in increasing concentrations (Fig. 3.8). Whereas in the second set, 40 μM peptide was included under the same reaction condition. The results therefore clearly indicated that, like LdCyP and N-del CyP, Pep-CyP, a small decapeptide of the chaperone, was equally capable of reactivating the aggregated AdK enzyme even in presence of high concentration of ADP.

3.5.3. R147 mutation in the cyclophilin peptide, PeP-CyP147-156, diminished its AdK reactivating property:

PeP-CyP147-156 sequence: RHVVFGKVLD
Mutant peptide sequence: G*HVVFGKVLD

Figure 3.8: PeP-CyP reversed ADP-induced inhibition of AdK. In absence of chaperone peptide, with increasing concentration of exogenous ADP, AdK activity showed gradual inhibition (♦), whereas, in the presence of PeP-CyP, inhibitory effect appeared to have been withdrawn (●).
In order to pinpoint the amino acid residue responsible for AdK reactivating property of the PeP-CyP, Ado phosphorylation reaction by the purified enzyme in presence of the mutant peptide was carried out and compared with non-mutated peptide. Earlier, it was reported from our laboratory that arginine residue, located at the 147th position of LdCyP, is crucial for reactivation. Site-directed mutagenesis of R147 to uncharged amino acid residue alanine inhibited the process of AdK stimulation (332). This result led us to change the sequence of the PeP-CyP to produce mPeP-CyP where the N-terminal R147 was converted to G147. Determination of reactivation profile of AdK upon conversion of R → G was then tested. Under standard radiochemical assay condition, the specific activity of Ado phosphorylation was monitored in the presence of wild type PeP-CyP and the mutated Pep-CyP (Fig 3.9). Results clearly showed that with increasing concentrations of chaperone peptide there was gradual increase in the yield of AMP upto certain concentration of the peptide after which the reaction reached a plateau phase (■). Whereas, the mutated PeP-CyP was completely ineffective in reactivating the enzyme (♦).

![Graph](Figure 3.9: Mutated peptide failed to reactivate AdK. Ado phosphorylation reaction rate was increased in presence of increasing concentration of PeP-CyP (■) whereas reactivation of AdK was not detected in the presence of mPeP (♦) under similar reaction conditions. AdK concentration was 50 nM.)
Conclusion:

The important points revealed from the studies presented in this chapter are summarized as follows:

1. The ADP produced during the AdK reaction remained attached to the enzyme and helps in formation of reversible AdK aggregates. Removal of the bound ADP from the enzyme-aggregate resulted in disaggregation and reactivation of the enzyme. Moreover, LdCyP acts by removing ADP from the ADP-binding site of the enzyme thereby allowing PK to get easy access of ADP.

2. Reactivation of AdK by chaperone is inversely proportional with the size of ADP-induced aggregate. In other words, with the progress of the chaperone-dependent disaggregation process, extent of AdK reactivation increased proportionately.

3. The functional data supported the conclusion that, LdCyP-chaperone, by displacing ADP from the enzyme, prevents ADP-induced transition to β-sheet conformation which resulted in disruption of aggregates and consequent enzyme reactivation.

4. For reactivation to occur, residue 147-156 of LdCyP appeared to be responsible. Pep-CyP147-156, which is small decapeptide fragment of the chaperone, is as efficient as full-length LdCyP in stimulating the activity of the aggregated AdK of L. donovani.

5. The activity inhibition effect of LdAdK in the presence of external ADP was reversed in the presence of decapeptide PeP-CyP.

6. Results strongly suggested that arginine residue at 147th postion of LdCyP is extremely important for the chaperonic function. Mutation of R147→G of LdCyP abrogated the reactivation of AdK.