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# Cyclophilin-mediated reactivation pathway of inactive adenosine kinase aggregates

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ABSTRACT

Monomeric adenosine kinase (AdK), a pivotal salvage enzyme of the purine auxotrophic parasite, *Leishmania donovani*, tends to aggregate naturally or selectively in presence of ADP, leading to inactivation. A cyclophilin (LdCyP) from the parasite reactivated the enzyme by disaggregating it. We studied the aggregation pathway of AdK with or without ADP. Transmission electron microscopy revealed that ADP-induced aggregates, as opposed to annular or torus-shaped natural aggregates, were mostly amorphous with protofibril-like structures. Interestingly, only the natural aggregates bound thioflavin *T* with a $K_D$ of 3.33 μM, indicating cross β-sheet structure. Dynamic light scattering experiments indicated that monomers formed aggregates either upon prolonged storage or ADP exposure. ADP-aggregates were disaggregated by LdCyP with concomitant reactivation of the enzyme. The activity revived with decrease in the aggregate size. Displacement of ADP from the ADP-aggregated enzyme by LdCyP resulted in reactivation. CD-spectral studies suggested that, like the natural aggregates, ADP induced formation of β-sheet structure in the ADP-aggregates. However, unlike the natural aggregate, it could be reconverted to α-helical conformation upon addition of LdCyP. Based on the results, a regulatory mechanism through interplay of ADP and/or LdCyP interaction with the enzyme is envisaged and a pathway of AdK reactivation by LdCyP-chaperone is proposed.



## Introduction

It is now well established that the ability to form aggregates is not restricted to proteins associated only with conformational disorders [1]. Hence, mechanisms by which aggregation and disaggregation of various proteins occur under various stress conditions within the complex cellular environment is under continuing investigation [2–4]. For the first time, using heat-treated protein aggregates, *in vitro* experiments showed that Hsp104, together with Hsp70 and Hsp40, could disaggregate protein aggregates, thereby facilitating their refolding [5]. The process of disaggregation was shown to be strictly ATP-dependent. Since then, several other chaperone complexes, having the capacity to disrupt protein aggregates, have been discovered [6,7].

Morphological transformation of *Leishmania donovani*, a purine auxotrophic parasitic protozoan with dimorphic life-cycle is accompanied with changes in the level and activity of a large number of proteins and enzymes [8]. Apart from transcriptional regulation, various other mechanisms, as yet unclear, seem to play role during this process [9]. *L. donovani* adenosine kinase (AdK)[1], which in presence of ATP catalyses phosphorylation of Ado to AMP, is one of such key enzymes of leishmanial purine salvage pathway [10]. The pivotal role of AdK in Ado homeostasis and in maintenance of intracellular AMP/ADP level is well known [11,12]. It has been shown that the increased rate of Ado utilization by the intracellular amastigotes, as opposed to extra cellular promastigotes of *leishmania*, is accompanied with parallel increase in the adenosine kinase specific activity, meaning that the activity increase was not due to increase in the content of the protein [13].

The roles of cyclophilins (CyPs), a multigenic family of ubiquitous proteins known for their cyclosporin A (CsA)-inhibitable intrinsic peptidyl prolyl *cis–trans* isomerase (PPIase) activity ranging from cell division, receptor maturation, protein folding etc. is well-known [14–17]. Hence, different strategies for designing of inhibitors against CyP as the new target for therapeutic intervention are emerging [18]. We earlier showed that AdK, which forms inactive soluble aggregates, could be disaggregated and reactivated by a CyP (LdCyP) from *L. donovani* [19,20]. This CsA-insensitive reactivation was observed *in vitro* in presence of stoichiometric amount of LdCyP. It was further observed that removal of ADP, one of its reaction-products, from the reaction mixture, led to increase in rate of the enzyme reaction [21]. However, in absence

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[1] *Abbreviations used:* AdK, Adenosine kinase; ER, Endoplasmic reticulum; Ni-NTA, Nickel-nitrilotriacetic acid; CsA, Cyclosporin A; ThT, Thioflavin T; TEM, Transmission electron microscopy; DLS, Dynamic light scattering; Ado, Adenosine.

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of any direct evidence, correlation between LdCyP-mediated disaggregation of AdK leading to its reactivation, remained ill-understood.

Because, phosphorylation of Ado to AMP by AdK is the driving force for the uptake of the purine base, especially in purine auxotrophs, the activity status of AdK under changing physiological condition of the organism may have bearing on the Ado uptake by the parasite [11]. Combining biophysical analysis with biochemical experiments we herein show that AdK, depending on the presence or absence of one of its reaction products, ADP, forms two types of aggregates. Whereas ADP-induced aggregates are disaggregated and reactivated by LdCyP-chaperone, the natural aggregates are resistant to disaggregation. Interestingly, the disaggregation of AdK by LdCyP is ATP-independent. The activity of the enzyme increases with the decreasing size of the ADP-induced aggregates.

## Materials and methods

### *Materials*

Unless otherwise mentioned, all reagents were of analytical grade and were purchased from Sigma Chemicals. N-terminal His-tag expression vector (pQE30) and Ni–NTA-agarose resin were the products of Qiagen.

### *Expression and purification of LdCyP and AdK*

Procedures for expression and purification of LdCyP and AdK were essentially same as described elsewhere [20]. Homogeneity of purified enzyme and LdCyP was routinely checked by SDS–PAGE analysis.

### *Size exclusion through HPLC for separating monomers from oligomers*

Due to inherent aggregating nature, the purified AdK had substantial population of aggregated multimers [20]. To separate monomers from the aggregated multimers, gel filtration of the purified enzyme using HPLC was carried out using the Biosuite-250 (7.5 × 300 mm, 13.25 ml, Waters, USA) column. The resolution range of the column was checked by employing standard markers, BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and lysozyme (14 kDa), eluted, respectively at retention times 14.7, 16.2, 21.5 and 23.1 min. Elution of blue dextran at 10 min was taken as the void volume. Purified AdK (100 μl, 4 mg/ml) was injected into the equilibrated column (20 mM Tris, pH 7.5, and 60 mM NaCl). Flow rate was maintained at 0.5 ml/min. The absorbance was monitored at 280 nm. AdK, eluting at retention times 10 and 18 min, corresponding to mixture of aggregates (void volume) and 38 kDa (monomeric), respectively were collected. The concentrations of the purified aggregates and monomers, following HPLC, were 20 and 184 μg/ml, respectively.

### *Thioflavin T fluorescence assay*

The concentration of thioflavin T (ThT), dissolved in water, was determined using extinction coefficient of 26,620 $M^{-1}$ $cm^{-1}$ at 416 nm [22]. The ThT assay buffer consisted of 20 mM Tris–HCl, pH 7.5 and 100 mM NaCl. Fluorescence emission of ThT-bound protein was scanned from 450 to 600 nm wavelength keeping 5 nm slit width using Hitachi F-4500 spectrofluorimeter. Fixing excitation wavelength at 440 nm, emission at 485 nm was monitored. Fluorescence contribution of the buffer was subtracted. Binding affinity ($K_D$) was calculated from the experimental results employing Klotz's equation, $1/r = (K_D/n)(1/[\text{free ThT}]) + 1/n$, where, $r$ = [bound substrate]/[total substrate] and n ~1 was calculated to be the number of ThT molecule bound per protein molecule [23].

### *Adenosine kinase reactivation assay*

The activity of AdK was measured at 30 °C by determining the formation of adenine mononucleotide from [2-$^3$H]-adenosine (Ado) in the presence of ATP and magnesium [10,24]. The concentrations of [2-$^3$H]-Ado (100 cpm/pmol) and ATP, in a 50 μl reaction mixture, were maintained at 50 μM and 1 mM, respectively. Increased efficiency of Ado phosphorylation by AdK was followed by adding LdCyP at indicated concentrations. AdK concentration was maintained at 50 nM. During reactivation assays, the concentration of protein substrate (AdK) was chosen to obtain the optimal extent of activation as measured by AMP formation. For product inhibition studies with ADP, assays were performed for 15 min at 30 °C in the presence of 400 μM ATP.

### *Isolation of aggregated ADP-enzyme complex*

ADP-aggregate was isolated using two different ways. In the first method, AdK-monomer (200 μl, 184 μg/ml) was incubated with Ado (66 μM) and ATP (1 mM) under enzyme-assay condition for 60 min at 30 °C. Under these conditions, the enzyme became completely inactive after 60 min [20].Whereas the second method involved direct incubation of the AdK-monomer with ADP (100 μM) for 15 min. In both instances, the incubated reaction mixtures were passed through the same gel-filtration column and the aggregated enzyme eluting at 10 min retention time (void volume) was collected. This step completely removed all the small unbound molecules like Ado, ATP, ADP, AMP, $Mg^{++}$ and monomeric AdK. This aggregated enzyme was analysed for the presence of ADP, reactivation in presence of LdCyP and for TEM experiments.

### *ADP removal from the ADP-bound enzyme aggregate*

To study the effect of LdCyP on the release of ADP from the void-peak eluted ADP-bound enzyme aggregate, a two-pronged approach was adopted. Using the ADP-enzyme aggregate, two sets of experiments were carried out. In the first set, the enzyme-aggregate was used as the source of the enzyme for activity analysis in presence or in absence of LdCyP whereas in the other set the enzyme-aggregate complex was assayed for the presence of ADP bound to the enzyme. In the first set, a fixed amount of the ADP-bound aggregated enzyme was treated separately for 30 min in presence of (i) assay buffer as control, (ii) LdCyP (15 μM), (iii) phosphoenolpyruvate (PEP)(1 mM) and pyruvate kinase (PK) (40 IU/ml), (iv) PEP and (v) PK. Using a fixed aliquot (10 μl) from these separately treated enzyme, kinetics of [$^3$H]-Ado phosphorylation was followed and [$^3$H]-AMP formed at indicated time intervals was determined. PK, in presence of saturating concentration of PEP and optimal $Mg^{++}$, converts ADP to ATP. In the second set, to measure (i) the ADP-content in the ADP-enzyme aggregate and to determine (ii) the effect of LdCyP on the release of ADP from the said complex, PK/LDH (lactate dehydrogenase)-coupled NADH (reduced nicotinamide adenine dinucleotide) oxidation assay was carried out with or without LdCyP [10]. The assay, carried out in presence of $Mg^{++}$, PEP and PK, spectrophotometrically monitored drop in absorbance of NADH at $A_{340\ nm}$. For ADP measurement, the amount of ADP-enzyme complex used was 5–10-fold higher over the first Ado-phosphorylation assay and lacked Ado and ATP. Calculations were as per the equation, A = Ecl, where A; absorption difference, E; extinction coefficient, c; concentration of protein and l; path-length of the cuvette. According to the principle of the PK/LDH-coupled NADH oxidation assay, the amount of ADP converted to ATP is equivalent to NADH. Extinction coefficient (E) of NADH at 340 nm was taken as 6220 $M^{-1}$ $cm^{-1}$. Protein determination was by Bio-Rad.

### *Dynamic light scattering (DLS) experiments*

Hydrodynamic diameters of AdK (2 μM), in absence and in presence of LdCyP (2 μM) and/or ADP (100 μM), was measured using