

Research Article

## CHAPERONE ACTION OF CYCLOPHILIN ON LYSOZYME AND ITS AGGREGATE

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**Abstract:** Various protein aggregates are formed in cellular systems when partially misfolded/folded states of proteins are trapped in a certain conformation. It eventually leads to the formation of larger and more stable aggregates. In the aggregated state proteins are functionally inactive and they are often enriched with antiparallel  $\beta$ -strands as compared to the native state. It has been established that a closely related molecular chaperone activity helps many proteins to preserve their cellular function by maintaining the protein conformation in their functional states. Thus the action of chaperone helps the cell to sustain various stresses that otherwise cause an extensive protein denaturation and aggregation. Recently, our laboratory demonstrated that AdK which has an inherent tendency to form inactive soluble aggregates could be disaggregated by cyclophilin derived from *L. donovani* (LdCyp) in an isomerase-independent fashion and resulted reactivation. Investigation is continuing to evaluate its action on amyloid aggregates formed by lysozyme. In addition the self and co-aggregation process of cyclophilin has been studied by fluorescence and TEM measurements. To our surprise, we observed the formation of elongated and fine fibrils when lysozyme was co-incubated with cyclophilin. Cyclophilin itself did not form any detectable aggregates under similar condition; however, it showed potential to disaggregate amorphous aggregate of lysozyme. Computational analysis was further performed to describe the aggregation and disaggregation of the proteins and their amyloidogenic behavior.

**Keywords:** cyclophilin; fluorescence; aggregate; lysozyme; chaperone.

### Introduction

It is now well established that a large number of human diseases arise from the failure of a particular protein/peptide to adopt and/or to remain in its functional conformational state (Stefani, 2004). These pathological conditions are often described as protein conformational disorder/diseases (Fink, 1999; Stefani and Dobson, 2003). Instability of the proteins/peptides to remain in its native functional states due to cellular and other external forces leads to a reduction in the quantity of active protein to

render their function and the instability often leads to misfolding of the proteins that contribute to the formation of unwanted protein aggregates (Jaenicke and Seckler, 1999).

Hence, the mechanisms by which aggregation and disaggregation of various proteins occur in its functional form, in different cells and in solution condition is under continuing investigation (Diamant *et al.*, 2000; Glover and Lindquist, 1998; Maiti and Maiti, 2012). To keep proteins in their native functional form, many other molecules act like chaperones by adding enough stability and folding cooperativity. *In vitro* experiments established that the combined action of chaperone molecules such as Hsp104, Hsp70 and Hsp 40, could disaggregate protein aggregates. Several other chaperone complexes,

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having the capacity to disrupt protein aggregates have been discovered (Dobson and Karplus, 1999; Fink, 1999). Thus the chaperone like action could facilitate refolding of proteins from their misfolded state in the aggregate (Bukau and Horwich, 1998). Protein adenosine kinase (AdK) which forms inactive soluble aggregates, could be disaggregated and reactivated by a Cyp (LdCyp) from *L. donovani* (Baker *et al.*, 1994; Nadeau *et al.*, 1993).

Cyclophilins (Cyps) are 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 (Liu *et al.*, 1992; Schreiber, 1991). Some cyclophilins are found to associate with well known chaperones (Andreeva *et al.*, 1999). It is observed that the transcription of some cyclophilin genes is increased in heat shock condition. All these phenomena indicates its chaperone like action. Cyclophilin showed peptidyl prolyl isomerase activity and thus catalyzes the isomerization of peptide bonds from trans to cis form at proline residues. This has important effect on protein folding and facilitates structural organization and folding (Ansari *et al.*, 2002; Schiene and Fischer, 2000). It was found in an *in vitro* experiment that Cyp could help the reactivation of AdK oligomers and it leads to increase in the rate of the enzyme reaction (Chakraborty *et al.*, 2002; Dutta *et al.*, 2001; Handschumacher *et al.*, 1984; Liu *et al.*, 1992; Schreiber, 1991; Sen *et al.*, 2006). However, in absence of any direct evidence, the mechanism of LdCyp-mediated disaggregation of AdK and the reactivation of the enzyme remained largely unknown.

We combined fluorescence, aggregation assays, and bioinformatics approaches to study the action of LdCyp on the formation and disaggregation process of protein aggregates which are implicated in neurological disorder. LdCyp itself showed very weak/no tendency to form amyloid fiber in micromolar concentration range. However co-incubation of the protein with lysozyme resulted the formation of fine protein fiber. Kinetics of aggregate formation/dissociation and surface property of the

aggregates were discussed and the sequence aspects of both the proteins were analyzed to realize their interaction pattern.

## Materials and Methods

**Materials** - Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma-Aldrich Chemicals. Ni-NTA-agarose resin was purchased from Qiagen. Sequences of each of the proteins were retrieved in FASTA format from PDB database.

**Expression and purification of LdCyp** - Recombinant cyclophilin (LdCyp) was expressed in *Escherichia coli* strain M15. pQE32 vector containing the gene Cyp expressed in *E. coli* cell was streaked on the LB agar plates and kept in 37°C incubator-shaker for overnight. A single colony was isolated and subjected to LB media for the growth of primary culture until absorption at 600 nm was ~0.3. The primary culture was subjected to TB media at 37°C incubator-shaker for 3 hrs. This secondary culture was induced with 0.5 mM IPTG. Optimization of IPTG induction time was done by TCA precipitation. Bulk culture was prepared using the same protocol with 4 h incubation for IPTG induction. Induced cells were harvested and subjected to lysis using buffer containing 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8.0), 300 mM NaCl, 10 mM imidazole and 1 mg/mL lysozyme solution. The resultant suspensions were sonicated (RivotekSonicator) with a pulse of 15 sec on/ 15 sec off until proper homogeneity was obtained and centrifuged (Sorvall RC 6+) at 14000 rpm for 30 min at 4°C. The supernatants were directly loaded with pre-equilibrated Ni-NTA-agarose column. The column was washed with the buffer containing 20 mM imidazole and the bound protein was subsequently eluted with 250 mM imidazole buffer. Imidazole-eluted His<sub>6</sub>-tagged proteins were extensively dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5) and 1% glycerol. Presence of the purified protein was confirmed by 15% SDS-PAGE band.

**Absorption measurements** - To determine the protein concentration, UV-Vis absorbance measurement of the proteins were done at 280 nm using UV-Vis spectrophotometer; (UV-2401PC, Shimadzu).

**Fluorescence measurements** - Fluorescence measurements were made on a Varian Cary Eclipse fluorimeter by exciting the samples at 440 nm and scanning the emission wavelength from 450 to 600 nm. The slit width was kept 2.5 nm for excitation and 5 nm for emission. Fluorescence intensities were recorded at the maximum of the emission band at 485 nm. A stock solution (1 mM) of ThT was prepared in double distilled water and kept in dark at 4°C. Prior to experiment, the exact concentration was determined by UV-absorbance of the filtered (using 0.22 micron PVDF filter) and diluted (in ethanol) stock solution using an extinction coefficient of  $26,620 \text{ M}^{-1}\text{cm}^{-1}$  at 416 nm. For aggregation experiments, 500  $\mu\text{M}$  lysozyme was incubated in 10 mM HCl (pH 2) (Frare *et al.*, 2004) and LdCyp was incubated in phosphate buffer (25 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 0.02%  $\text{NaN}_3$  solution, pH 7.4) at 37°C/65°C. ThT assays were performed in triplicate to measure fibril formation using appropriate volume of incubated sample and 25  $\mu\text{M}$  ThT in 50 mM glycine•NaOH, pH 8.5. Samples were excited at 440 nm and emission at 485 nm was measured. To monitor the kinetics of the disaggregation of lysozyme aggregate by the chaperone (LdCyp), thioflavin T fluorescence spectroscopy was studied at different time intervals. Fluorescence intensities at 485 nm were plotted as a function of time after subtraction of the buffer signal.

**Transmission electron microscopy (TEM)** - For electron microscopy, 10  $\mu\text{l}$  of the incubated sample was taken after 160 hrs, placed on carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA) for 10 min, and stained with 2% (w/v) uranyl acetate for 1 min. Images were taken on FEI Tecnai, G2 spirit Biotwin electron microscope.

**Calculating percentage of disorder** - Structural disorder was calculated using IUPred algorithm (Dosztanyi *et al.*, 2005) which is available at <http://iupred.enzim.hu>. The percentage of disorder for each protein was estimated by counting the number of disordered amino acids as predicted by IUPred and it was divided by the length of the protein sequence followed by multiplication with 100.

**Calculating AR** - Amyloidogenic regions of the proteins were identified by a web based

computational tool Waltz (Maurer-Stroh *et al.*, 2010) (<http://waltz.switchlab.org>). The waltz method uses a position specific scoring matrix (PSSM) to determine the amyloid-forming segments (Maurer-Stroh *et al.*, 2010). The database was trained from a large set of experimentally characterized amyloid forming peptides.

**Calculating LCR** - The content of LCR of an individual protein was predicted by SEG methods (Wootton, 1993) as implemented in SMART (Schultz, 1998), a web based server available at <http://www.smart.embl-heidelberg.de>. Default SEG parameters were used for finding LCRs. The SEG method detects LCRs based on the measurement of information content present in the complexity state vector.

**Prediction of secondary structure** - APSSP2 was used for the secondary structure prediction of each protein from their aa sequence (Raghava, 2000). The algorithm uses a sequence of amino acids as a query sequence input and predicts the corresponding secondary structure with certain confidence level. Percentages of residues that prefer to be in helical,  $\beta$ -strand and coiled conformation in the predicted secondary structure of a particular protein were calculated by taking a ratio of the total residues in a particular conformation to the length (total number of amino acids).

## Results and Discussion

LdCyp has some chaperone activity and could disaggregate oligomers of adenosine kinase. In the current investigation we showed the possible interaction of cyclophilin (LdCyp) with lysozyme and its aggregate. Figure 1 shows the ribbon representations of the PDB structure of both the proteins. The crystal structure information indicated that the secondary structure composition of cyclophilin was of 15%  $\alpha$ -helix, 31 %  $\beta$ -sheet, and 54% others and 18.18%, 29.41% and 51.34% respectively as predicted by APSSP2. Similarly, crystal structure analysis indicated content of different amount of secondary structure present in lysozyme, i.e., 38%  $\alpha$ -helix, 10%  $\beta$ -sheet, and 52% others, whereas, as per APSSP2, the composition is 39.53%, 9.3% and 49.61% respectively (Table 1). Figure 2 details the individual protein sequences. Using the protein

sequences possible presence of structural disorder was calculated by computational algorithm IUPred. The analysis indicated that both the proteins contained no measurable disordered regions in their protein sequences. The obtained data are presented in Table 2.

A major fraction of the residues was hydrophobic and Asn (N) was the most abundant (10.85%) residue in lysozyme and 5.35% in *L. donovani* cyclophilin respectively. Major residues in the lysozyme were Ala (A) and Gly (G) (9.30%

each), Arg (R) (8.53%), Ser (S) (7.75%), Lys (L) and Cys (C) (6.20%). The aa residues in LdCyp were depleted in 6.95%, 10.16%, 3.74%, 4.28%, 7.49%, 1.07% respectively (Table 3, Figure 3). Previous investigation by homology modeling studies indicated that the exposed hydrophobic residues of LdCyp may interact with solvent-accessible hydrophobic surface of AdK and helps its reactivation. A similar interaction may be possible by the hydrophobic action of both the molecules, lysozyme and LdCyp.

**Table 1**  
**Secondary structure composition**

Protein	PDB data % $\alpha$ -helix	APSSP2 data % $\alpha$ -helix	PDB data % $\beta$ -sheet	APSSP2 data % $\beta$ -sheet	PDB data % other	APSSP2 data % other
Cyclophilin	15	18.18	31	29.41	54	51.34
Lysozyme	38	39.53	10	9.3	52	49.61

**Table 2**  
**AR and LCR in cyclophilin (LdCyp) and lysozyme**

Protein	Seq length	% disorder	% res in AR	% res in LCR	AR	LCR
Cyclophilin (LdCyp)	187	0	11.76	12.3	AKVYFD(27-32); VIQNFMIQ(79-86); SQFFITTA (132-139)	VAVLAVVLAL (4-14); GGDFTNFDGTG (87-98)
Lysozyme	129	0	13.18	0	FESNFNTQATN (34-44); IRGCRL (124-129)	-

**Table 3**  
**Amino Acid Residue Percentage of the Proteins**

1-letter amino acid code	Lys (no. of aa residue)	% aa	Cyp (no. of aa residue)	% aa
N	14	10.85271	10	5.347594
A	12	9.302326	13	6.951872
G	12	9.302326	19	10.16043
R	11	8.527132	7	3.743316
S	10	7.751938	8	4.278075
C	8	6.20155	2	1.069519
L	8	6.20155	14	7.486631
D	7	5.426357	11	5.882353
T	7	5.426357	13	6.951872
I	6	4.651163	10	5.347594
K	6	4.651163	11	5.882353
V	6	4.651163	19	10.16043
W	6	4.651163	1	0.534759
F	3	2.325581	15	8.02139
Q	3	2.325581	4	2.139037
Y	3	2.325581	3	1.604278
E	2	1.550388	9	4.812834
M	2	1.550388	5	2.673797
P	2	1.550388	8	4.278075
H	1	0.775194	5	2.673797



AR acts as a key for several protein aggregations and amyloid fibril formation. We detected ARs by using Waltz algorithm. Waltz detected amyloidogenic region in both the protein sequence and thus the proteins were found to be amyloidogenic and each of these proteins contained at least one AR. AR regions found in lysozyme were FESNFNTQATN (34-44) and IRGCRL (124-129). LdCyp contained three amyloidogenic regions, AKVYFD (27-32), VIQNFMIIQ (79-86), SQFFITTA (132-139). The total AR sequence in lysozyme was 13.18% of the total protein sequence. The content of the AR sequence in cyclophilin was about 11.76% of the total protein sequence (Table 2, Figure 2).

We further determined the complexity pattern of the sequences in the protein and the ARs as its presence could modulate the aggregation process (Dobson and Karplus, 1999). The sequence complexity was analyzed using the SEG algorithm as implemented in the SMART server (Schultz, 1998). Percentage of sequences in the low complexity region (LCR) in each protein are given in Table 2. The content of the LCR sequence (%) was higher in LdCyp than the lysozyme.

The presence of AR regions in protein sequence often indicates the possible tendency of the proteins to form amyloid aggregates. We have found similar % of AR sequence both in the LdCyp and lysozyme; it indicates that both the protein could have similar probabilities of formation of amyloids, however, lysozyme found to form aggregates in acid denatured condition. The presence of a low complexity region in cyclophilin may prohibit hydrophobic association of the residues to form nuclei that leads to formation of elongated protein fiber. Sequence analysis of LdCyp in comparison to lysozyme also indicated more hydrophobic residue; it indicated that it would form aggregate more efficiently than the lysozyme. However, detailed temperature dependent study and pH effects may in future give better understanding of why cyclophilin fails to form aggregates, although it contains more  $\beta$ -sheet structural component (Table 1).

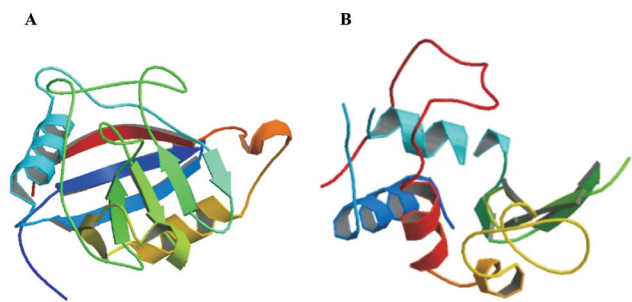
To better understand the aggregation and the disaggregation process we *performed in vitro* experiments. Recombinant cyclophilin (LdCyp)

was expressed in *Escherichia coli*. Figure 4A depicts the optimization time for IPTG induction. Presence of the purified protein was confirmed by 15% SDS-PAGE band (Figure 4B).

Possible formation of aggregation and amyloid like fibril was performed by incubation of cyclophilin at 50  $\mu$ M concentrations for several days and the formation of self aggregation and amyloid type of aggregation was monitored by ThT fluorescence assay. Even after incubation for a week at 37°C and at 65°C independently, we could not find any enhancement of the fluorescence intensity (Figure 5). It indicated that the LdCyp was not favoring fibril formation though it contained three amyloidogenic regions. It could be due to the fact that the regions were not well exposed and the structural strain prohibited the aggregation formation.

Lysozyme (500  $\mu$ M) was incubated for more than seven days at pH 2.0 and 7.4, and temperatures at 37°C and 65°C to induce aggregation and fiber formation. The extent of fibril formation was monitored by Thioflavin-T fluorescence measurement. Protein fibrillogenesis was evaluated at a protein concentration of 5  $\mu$ M in 25 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  (pH 7.4), 0.02%  $\text{NaN}_3$  solution. Samples were incubated at 37°C in upright 1.5 mL Eppendorf tubes using an Eppendorf incubator. Thioflavin T fluorescence was measured at selected time intervals and we observed amyloid type of aggregates. TEM analysis was performed to confirm aggregate morphology and it was not smooth and mostly amorphous (Figure 6A).

To realize the lysozyme-LdCyp interaction and to probe how LdCyp affects the stability of preformed lysozyme aggregates, we carried out ThT assays of the aggregated solution mixed with LdCyp (Figure 6B). A significant effect of LdCyp on the aggregates was reflected on ThT fluorescence. The lysozyme aggregates mixed with LdCyp showed immediate decrease in ThT fluorescence. Longer incubation caused further decrease in the ThT fluorescence although to a smaller extent (Figure 7). This study indicated that the LdCyp could cause disaggregation of preformed aggregates of other proteins.



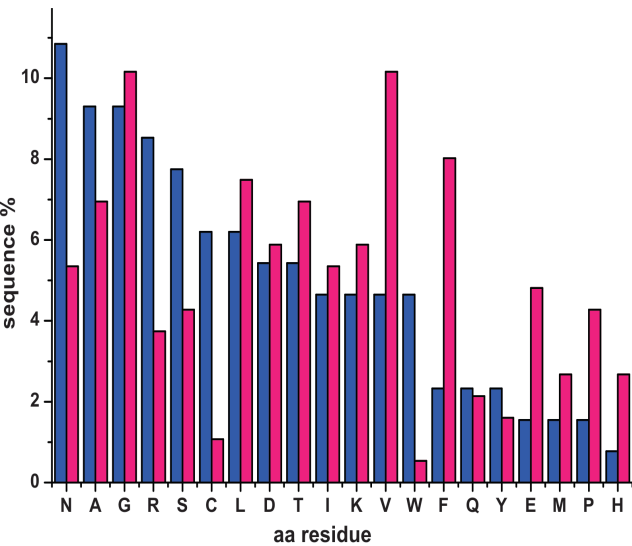
**Figure 1:** Ribbon representation of the PDB structure of cyclophilin and lysozyme. A: *Leishmania donovani* Cyclophilin (PDB ID: 2HAQ), B: Lysozyme (PDB ID: 1LYZ).

M	R	F	V	A	V	L	A	V	V	L	C	A	L	S	F	L	N	V	A
A	E	P	E	V	T	A	K	V	Y	F	D	V	M	I	D	S	E	P	L
G	R	I	T	I	G	L	F	G	K	D	A	P	L	T	T	E	N	F	R
Q	L	C	T	G	E	H	G	F	G	Y	K	D	S	I	F	H	R	V	I
Q	N	F	M	I	Q	G	G	D	F	T	N	F	D	G	T	G	G	K	S
I	Y	G	E	K	F	A	D	E	N	L	N	V	K	H	F	V	G	A	L
S	M	A	N	A	G	P	N	T	N	G	S	Q	F	F	I	T	T	A	P
T	P	W	L	D	G	R	H	V	V	F	G	K	V	L	D	G	M	D	V
V	L	R	I	E	K	T	K	T	N	S	H	D	R	P	V	K	P	V	K
I	V	A	S	G	E	L													

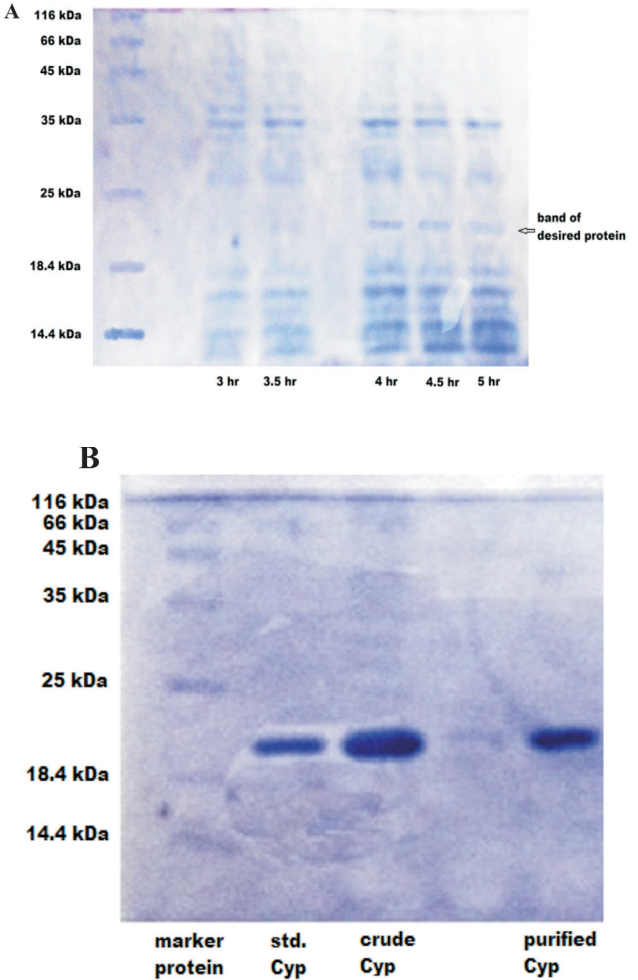
B

K	V	F	G	R	C	E	L	A	A	A	M	K	R	H	G	L	D	N	Y
R	G	Y	S	L	G	N	W	V	C	A	A	K	F	E	S	N	F	N	T
Q	A	T	N	R	N	T	D	G	S	T	D	Y	G	I	L	Q	I	N	S
R	W	W	C	N	D	G	R	T	P	G	S	R	N	L	C	N	I	P	C
S	A	L	L	S	S	D	I	T	A	S	V	N	C	A	K	K	I	V	S
D	G	N	G	M	N	A	W	V	A	W	R	N	R	C	K	G	T	D	V
Q	A	W	I	R	G	C	R	L											

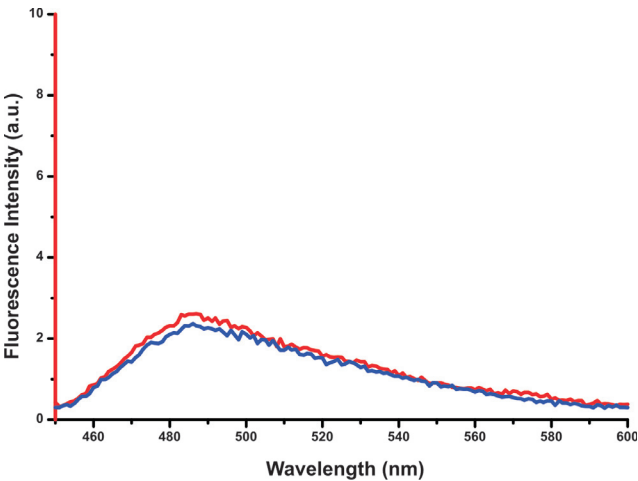
**Figure 2:** Sequence of cyclophilin and lysozyme. A: *Leishmania donovani* Cyclophilin (LdCyp), B: Lysozyme, AR and LCR of the respective proteins are shown in the following color code, AR-   , LCR-   .



**Figure 3:** Amino acid residue percentage (%) distribution plot, Lysozyme (Lys) vs. Cyclophilin (LdCyp). Proteins are marked in the following color code, Lysozyme-   , LdCyp-   .

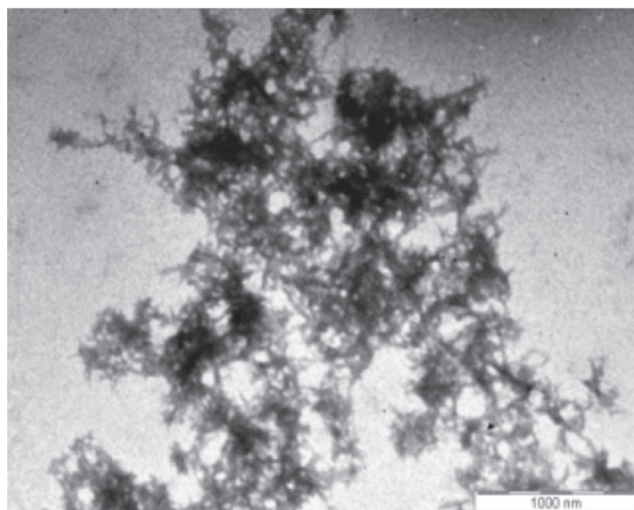


**Figure 4:** Expression and purification of the protein (SDS-PAGE band). A: SDS-PAGE after 10% TCA precipitation, B: SDS-PAGE of purified LdCyp.

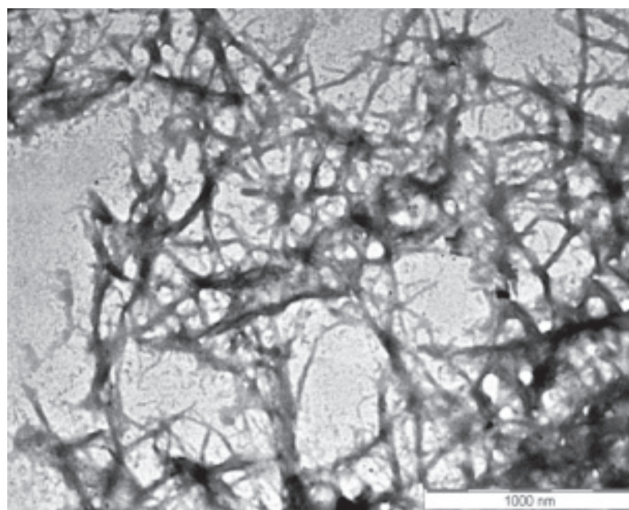


**Figure 5:** ThT fluorescence of Cyp incubated at 37 °C for 160 hrs. Color codes are, ThT assay buffer -   , LdCyp-   . (ThT fluorescence of Cyp incubated at 65 °C is not shown here since no change in ThT intensity was observed).

A

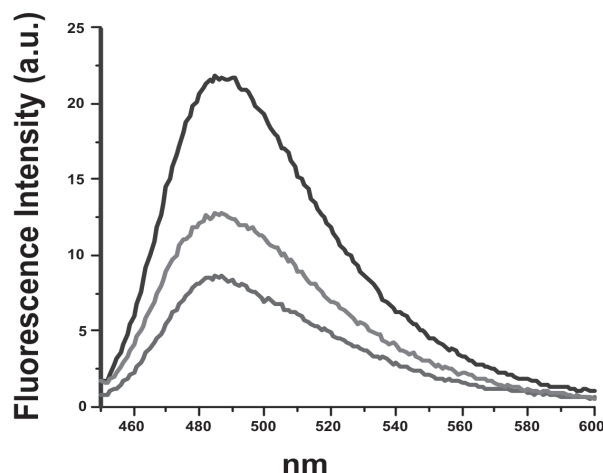


B

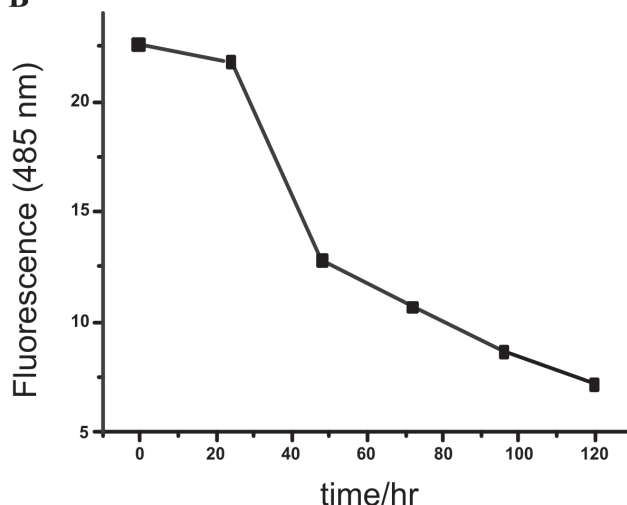


**Figure 6:** TEM images of protein aggregation. A: Lysozyme aggregate (diameter of fiber is ~5-10 nm), B: Lysozyme aggregate in the presence of cyclophilin (diameter of fiber is ~10-50 nm)

A



B



**Figure 7:** ThT fluorescence of Lysozyme in presence of *Leishmania donovani* Cyclophilin at different time interval. A: ThT fluorescence; colour codes implicate the time intervals of the incubation of lysozyme and LdCyp at 37 °C, ■ - after 24 hrs, ■ - after 48 hrs, ■ - after 96 hrs; B: Scattered plot of ThT fluorescence intensity at 485 nm at different time interval.

Co-Incubation of lysozyme and LdCyp indicated, however, a formation of smooth and very compact amyloid-like protein fiber. It indicated that the LdCyp could remove and prohibit the formation of an amorphous type of aggregates of lysozyme. It may have very little effect on very compact protein fiber.

### Summary

Computationally it was confirmed that cyclophilin from *L. donovani* (LdCyp) contained amyloidogenic region and also regions which

shows low sequence complexity. It was expected that this protein may form fibrillar aggregates. However, the protein failed to produce any detectable aggregates in a solution condition that promote formation of amorphous aggregates of lysozyme. LdCyp could disaggregate the amorphous aggregate to a certain extent indicating LdCyp's unique ability to interact with amorphous aggregates or prefibrillar assembly structures. It was shown earlier that LdCyp could reactivate oligomeric AdK. Here, we showed that LdCyp may have unique properties to



disaggregates amorphous or oligomeric aggregates made of any other protein. More experiments and analysis are currently continuing to address this issue and the reason behind the stability of cyclophilin and not the formation of aggregates in a similar solution condition where lysozyme with less number of  $\beta$ -sheet structural component forms aggregates.

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### Abbreviations

Aa, amino acid; APSSP2, advanced protein secondary structure prediction server; AR, amyloidogenic region; Cyp, cyclophilin; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; LB, luria broth; LCR, low complexity region; LdCyp, *Leishmania donovani* cyclophilin; Lys, lysozyme; Ni-NTA, nickel-nitrilotriacetic acid; PDB, protein data bank; SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis; SMART, simple modular architecture research tool; TB, terrific broth; TEM, transmission electron microscopy; ThT, thioflavin-T.

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