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Novel protein–protein interaction between spermidine synthase and S-adenosylmethionine decarboxylase from *Leishmania donovani*

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ABSTRACT

Polyamine biosynthesis pathway has long been considered an essential drug target for trypanosomatids including *Leishmania*. S-adenosylmethionine decarboxylase (AdoMetDc) and spermidine synthase (SpdSyn) are enzymes of this pathway that catalyze successive steps, with the product of the former, decarboxylated S-adenosylmethionine (dcSAM), acting as an aminopropyl donor for the latter enzyme. Here we have explored the possibility of and identified the protein–protein interaction between SpdSyn and AdoMetDc. The protein–protein interaction has been identified using GST pull down assay. Isothermal titration calorimetry reveals that the interaction is thermodynamically favorable. Fluorescence spectroscopy studies also confirms the interaction, with SpdSyn exhibiting a change in tertiary structure with increasing concentrations of AdoMetDc. Size exclusion chromatography suggests the presence of the complex as a hetero-oligomer. Taken together, these results suggest that the enzymes indeed form a heteromer. Computational analyses suggest that this complex differs significantly from the corresponding human complex, implying that this complex could be a better therapeutic target than the individual enzymes.

1. Introduction

Polyamines are small ubiquitous basic molecules that play multiple essential roles in cell development and proliferation in all classes of organisms. The first step of polyamine biosynthesis involves the synthesis of ornithine from arginine by arginase which is subsequently decarboxylated to the diamine putrescine by ornithine decarboxylase. Putrescine is further converted to spermidine and spermine through the sequential addition of aminopropyl residues by spermidine and spermine synthases respectively [1,2]. The latter two reactions of this pathway involve decarboxylated S-adenosylmethionine as an aminopropyl group donor, produced by S-adenosylmethionine decarboxylase (AdoMetDc) [3].

The importance of the polyamine biosynthesis pathway is revealed by the fact that the knockouts of the ornithine decarboxylase or AdoMetDc genes in mouse are lethal at very early embryonic stages [4]. In *Leishmania*, ornithine decarboxylase, AdoMetDc and spermidine synthase (SpdSyn) knockout mutants have negligible ability to infect mouse [5,6]. Thus, polyamine biosynthesis pathway is essential for the viability, growth and infectious mammalian stage of the trypanosomatid parasite including *Leishmania donovani*, validating all the enzymes of this pathway as potential drug targets [7–10]. The known inhibitors of enzymes of polyamine biosynthesis pathway from trypanosomatids exhibit optimum drug-like properties. D,L-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase exhibits efficacy against *Trypanosoma brucei* infections in both mice and patients with late stage African sleeping sickness [11–13]. In addition, AdoMetDc inhibitors are also effectual antitrypanosomal agents [14–16].

The enzymes that accomplish sequential catalytic steps in any metabolic transformations are known to form complexes called metabolons [17]. This complex formation favors effective transport of substrates from the active site of one enzyme to the other and increases the local concentration of the substrate of second enzyme, ensuring a high catalytic rate. Metabolon formation is advantageous for unstable substrates as they are directly transferred to the active site of the enzyme. Here in this study we report such a metabolon formation of two successive enzymes of polyamine biosynthesis pathway of *L. donovani*, spermidine synthase (SpdSyn) and S-adenosylmethionine decarboxylase (AdoMetDc). The interaction between these two proteins was identified and confirmed by GST pull down assays, and rationalized by means of biophysical, biochemical techniques.

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2. Materials and methods

2.1. Materials

Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, DNA Marker, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Fermentas International Inc. Anti-His and anti-GST antibodies were purchased from Sigma–Aldrich. Other chemicals used in this study were of the highest grade available.

2.2. Sequence analysis and database search

The coding sequence of *L. donovani* SpdSyn and AdoMetDc were retrieved from Uniprot database (http://www.uniprot.org/uniprot). The protein–protein interaction was predicted by STRING 9.0 (http://string-db.org) [18] which predicts interaction on the basis of genomic context, high-throughput experiments, co-expression, experiments and previous knowledge.

2.3. Cloning, over-expression and purification of SpdSyn and AdoMetDc

The 900 bp long *L. donovani* SpdSyn gene amplified from the genomic DNA of *L. donovani* using specific sense "5′GCT AGC ATG CCA GGC CCC GGT CTT CTG 3′ and anti-sense "5′GGA TCC CTC GTT CAG GTG AGC CGC GAA GC 3′ primers designed using Oligo software [19], with sites for NheI and BamHI restriction enzymes (underlined) respectively. Similarly the 1146 bp long AdoMetDc gene was amplified, from *L. donovani* genomic DNA by using specific sense "5′AGT GGA TCC ATG AAT GTC TGC TCG AAC ACC ACA 3′ and antisense "5′CTT AAG CTT GTC GGG CCC ACC CTC GGC GCC3′ primers having sites for BamHI and HindIII restriction enzymes (underlined) respectively. The amplified genes were cloned in T/A vector pTZ57R/T (InsTA clone™ PCR cloning kit, Fermentas International Inc.). LdSpdSyn was then sub-cloned downstream of the T7 promoter expression vector pET23a (Novagen) between NheI and BamHI sites. *Escherichia coli* C41 host cells were transformed with the recombinant plasmid pET23a–SpdSyn and used for over-expression. LdAdoMetDc was sub-cloned downstream of the T7 promoter expression vector pGEX-kg (Qiagen) between BamHI and HindIII sites. *E. coli* BL21 (DE3) host cells were transformed with the recombinant plasmid pGEX–AdoMetDc and was used for over-expression.

Luria–Bertani (LB) broth containing ampicillin (100 μg/ml) was inoculated with *E. coli* containing recombinant plasmid and cultured overnight at 37 °C. Fresh ampicillin containing LB broth was inoculated with 1:100 dilution of this seed culture and incubated at 37 °C to an OD_{600} of ~0.6. Over-expression was then induced by adding 1 mM isopropyl-1-thio-β-galactopyranoside (IPTG) and allowed to grow for 6 h at the same temperature. The culture was subsequently harvested by centrifugation, resuspended in a buffer containing 50 mM Tris–HCl pH 7.5 and 150 mM NaCl. Cells were lysed by sonication with a 10 s on and 10 s off pulse for 15 min. Cell debris was removed by centrifugation at 12,000 rpm for 30 min and the supernatants were loaded on IMAC columns pre-equilibrated with the same buffer. The column was incubated for an hour and subsequently washed with buffers containing 10 mM and 20 mM imidazole. Protein was then eluted with 400 mM imidazole in the same buffer.

The AdoMetDc–GST fusion protein in pGEX vector was purified by GST affinity chromatography. Lysate containing the overexpressed protein in buffer A (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM PMSF and 3 mM DTT) was incubated with GST matrix (Protino®) for four hours and then washed with 5 column volumes of buffer B (50 mM Tris–HCl pH 8.0, 1 M NaCl, 1 mM PMSF, 0.5% Triton X-100 and 5 mM DTT). Bound protein was then eluted with 10 mM reduced glutathione in buffer A. The eluted proteins were dialyzed overnight into 50 mM HEPES–Na pH 7.5, 50 mM NaCl and 3 mM β-me then concentrated using 10 kDa cutoff centricon (Amicon).

2.4. Protein–protein interaction study

Protein–protein interaction was studied using the following techniques.

2.4.1. GST pull-down assay

For GST pull-down assay the cell lysate containing AdoMetDc–pGEX protein was incubated with GST agarose for four hours and then allowed to pass through the column. The protein bound GST matrix was washed with 2-column volumes of buffer containing 50 mM Tris–HCl pH 8.0, 1 M NaCl, 1 mM DTT, 0.5 mM PMSF and 0.7% Triton X-100. GST matrix with bound AdoMetDc, was then incubated with the cell lysate containing SpdSyn–pET23a for 3 h. The GST matrix was then washed with 5 column volumes of buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM DTT, and 0.5 mM PMSF. All bound proteins were then eluted with 20 mM reduced glutathione in the same buffer and checked on a 12% SDS–PAGE, anti-His and anti-GST western blots. For control similar experiments were performed in which only GST protein was incubated with SpdSyn.

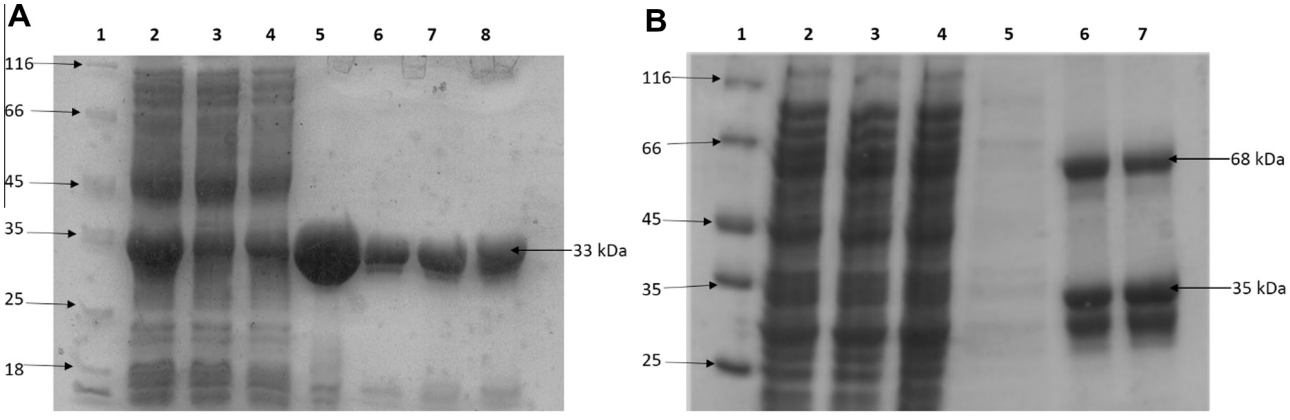

Fig. 1. SDS–PAGE analysis of SpdSyn (A) and AdoMetDc (B) during purification. Protein samples were analyzed on 12% SDS–PAGE and stained with coomassie blue. (A) Lane 1 – molecular weight marker, Lane 2 – induced cell lysate with 1 mM IPTG, Lane 3 – flow-through, Lane 4 – wash, Lane 5–8 purified protein after IMAC. (B) GST affinity purification of AdoMetDc. Lane 1 – protein marker, Lane 2 – load; Lane 3 – flow through; Lane 4 and 5 – wash; Lane 6 and 7 – eluted protein through GST purification.

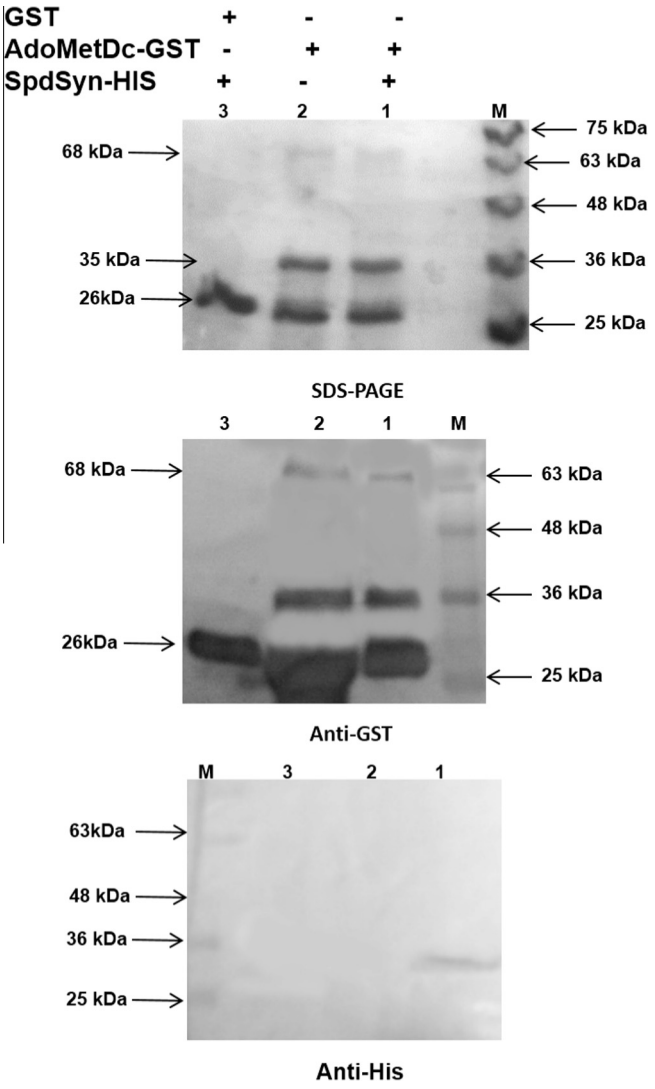

Fig. 2. The elution products of GST pull-down assay were analyzed on 12% SDS–PAGE (top panel): Lane M marker, Lane 1: elution of AdoMetDc–GST with SpdSyn. Lane 2: AdoMetDc alone, Lane 3: elution of GST only as negative control with SpdSyn. The band at ~68 kDa corresponds to full length AD-GST while the band at ~35 kDa corresponds to the autocatalyzed N-terminal fragment of AD (9 kDa) fused to SpdSyn (~33 kDa). To resolve this, the eluted products were then probed with anti-GST (middle panel) which shows similar results as SDS–PAGE. We confirmed interaction by anti-His (bottom panel) antibodies which show the presence SpdSyn protein (~33 kDa). The absence of a band corresponding to SpdSyn in Lane 3 in the anti-His blot confirms specific AdoMetDc–SpdSyn interaction.

2.4.2. Isothermal titration calorimetry

The thermodynamic parameters of the interactions between SpdSyn and AdoMetDc were determined by ITC (VP-ITC, MicroCal, Northampton, MA, USA). Both proteins were dialyzed in 50 mM HEPES–Na buffer pH 7.5, 50 mM NaCl and 10 μM AdoMetDc titrated against 100 μM SpdSyn. The thermograms were analyzed with Origin 7.0 software (MicroCal, USA) after subtraction of the thermogram for the buffer alone. Each thermodynamic parameter is the average of at least three independent measurements.

2.4.3. Fluorescence spectroscopic analysis of L. donovani SpdSyn–AdoMetDc interaction

LdSpdSyn and LdAdoMetDc were individually labelled with fluorescein isothiocyanate (FITC) dye which reacts with the primary amine groups in proteins. 2 μM of FITC-labeled sample was excited at 494 nm and the emission spectra recorded in the range of 500–600 nm, on a Perkin Elmer LS50b Luminescence Spectrometer, at 25 °C. The fluorescence spectra with increasing concentrations of the second unlabeled protein were measured and the change in FITC fluorescence was observed at 525 nm. Control experiments were performed in the same buffer, without the protein(s). The change in fluorescence was then related to the binding by the following equation [20,21]:

$$\Delta F/\Delta F_{max} = [\text{substrate}]_{tot}/(K_d[\text{substrate}]_{tot})$$

where ΔF is the magnitude of the difference between the observed fluorescence intensities in the presence and absence of the substrate at a given concentration of substrate, ΔF_{max} is the difference between the observed fluorescence intensities at zero and saturating substrate concentration, $[\text{Substrate}]_{tot}$, and K_f is the apparent dissociation constant. The K_d values were determined from a non-linear least-squares regression analysis of titration data. With all samples, fluorescence spectra were corrected for the background fluorescence of the solution. Deconvolution of curves was performed using the Prism software (Graph Pad software Inc.).

2.4.4. Size exclusion chromatography

Size exclusion chromatography was performed on the Superdex™ 200 10/300 prepacked column (manufacturer's exclusion limit 600 kDa for proteins) on an AKTA-FPLC (GE HealthCare Biosciences). The dialyzed and concentrated SpdSyn and AdoMetDc (1 mg/ml) was loaded onto the column pre-equilibrated with a buffer containing 50 mM HEPES–Na pH 7.5 and 50 mM NaCl. The elution was monitored using the absorbance at 280 nm. For interaction analysis both proteins were mixed in equal concentrations, incubated overnight and size exclusion chromatography profile was recorded. All measurements were made at 25 °C. Calibration of the column was performed using the low molecular weight standard kit (GE Healthcare) containing conalbumin, ovalbumin, carbonic anhydrase, RNAase and aprotinine as reference proteins.

3. Results

As mentioned earlier, enzymes that perform successive steps of a pathway tend to form a metabolon complex that results in the increased local concentration of the substrate thereby boosting the efficiency of the pathway. In *Arabidopsis thaliana*, it had been shown by yeast 2-hybrid complementation assay that spermidine synthase and spermine synthase form such a metabolon complex and it was also suggested therein that other enzymes in the pathway too might form metabolons [22]. The possibility of metabolon formation in the polyamine biosynthesis pathway might play an essential role in the regulatory mechanism, thus the detailed study of interaction between these two proteins is essential. With this in mind, we wondered if *L. donovani* AdoMetDc and SpdSyn too form such a complex. Preliminary computational protein–protein interaction studies, using web-based tools such as STRING 9.0, also seemed to favor the formation of such a complex, with a score of 0.984, which was higher than the score for *A. thaliana* SpdSyn and spermine synthase (0.924) and comparable with SpdSyn–ODC interaction (0.997) evident by positive genetic assay in yeast [23]. To explore this possibility, we have cloned, overexpressed and purified the two proteins and analyzed the interactions by various biophysical techniques.

3.1. Cloning and purification of L. donovani SpdSyn and AdoMetDc

L. donovani SpdSyn and AdoMetDc were cloned, overexpressed and purified by Immobilized metal affinity chromatography and Glutathione affinity chromatography respectively. Purified

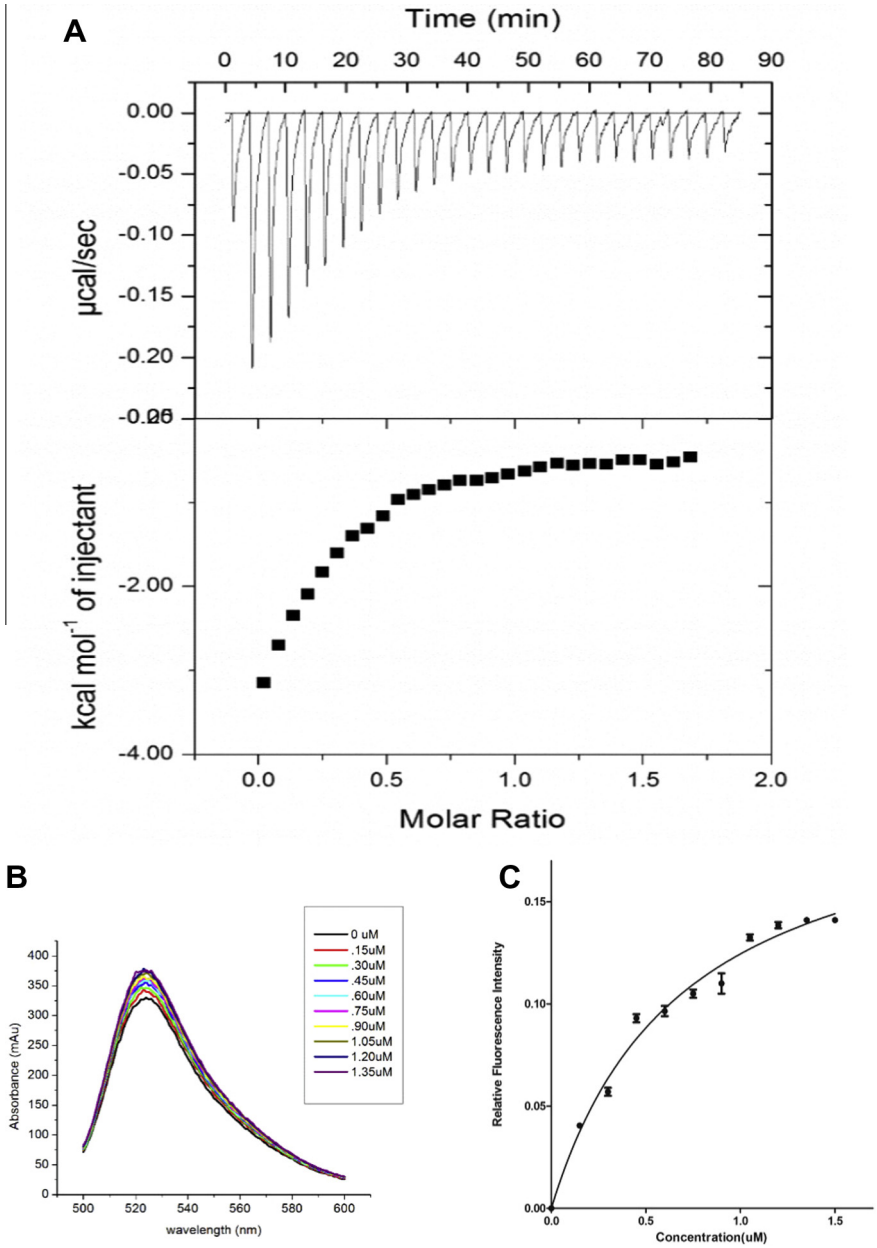

Fig. 3. Protein–protein interaction analysis by calorimetric and fluorescence spectroscopy. (A) Thermogram of binding of SpdSyn and AdoMetDc at 30 °C in HEPES–Na buffer pH 7.5. (B) Graph shows effect of increasing concentration of SpdSyn causes increase in fluorescence. (C) Saturation binding isotherm with dissociation constant 0.6 µM shows interaction between SpdSyn and AdoMetDc.

recombinant SpdSyn was observed as a single band corresponding to 33 kDa on a 12% SDS–PAGE (Fig. 1A), while recombinant AdoMetDc was observed as two bands, corresponding to the full length (68 kDa) AdoMetDc–GST fusion protein and a ~35 kDa fragment, the latter being the product after autocatalytic cleavage into mature α subunit of a 9 kDa along with 26 kDa N-terminal GST Tag and a β subunits of 33 kDa consistent with AdoMetDc from other trypanosomatids [1] (Fig. 1B). The autocatalysis does not require putrescine unlike human AdoMetDc, the result is consistent with earlier reports of Roberts *et al.* [24] and Velez, Brautigam and Philips [25].

3.2. Protein–protein interaction study

3.2.1. GST pull-down assay

The interaction between *L. donovani* SpdSyn and AdoMetDc was identified using GST pull-down assay. As mentioned in Section 2.4.1 the cell lysate containing AdoMetDc–pGEX protein was bound to a GST agarose column, upon which the cell lysate containing SpdSyn–pET23a was passed through. All bound proteins were then eluted with 20 mM reduced glutathione and run on SDS–PAGE (Fig. 2 top panel, Lane 1), which shows bands at ~68 kDa, corresponding to the full length GST–AdoMetDc, and at ~33–35 kDa, which could correspond to either the autocleaved N-terminal part of AdoMetDc–GST (35 kDa), or its C-terminal part (33 kDa) or SpdSyn (33 kDa). These products are not clearly delineated in this gel due to the similarity in their sizes, but have been confirmed with anti-GST and anti-His western blots (Middle and Bottom panels of Fig. 2). As a control, the SpdSyn containing lysate was also passed through the column incubated with GST alone (Lane 3), and here there are no bands corresponding to SpdSyn either in the gel or in the anti-His western blot, confirming that the interaction is indeed between AdoMetDc and SpdSyn. The experiment was also repeated without the addition of SpdSyn and is shown in Lane 2.

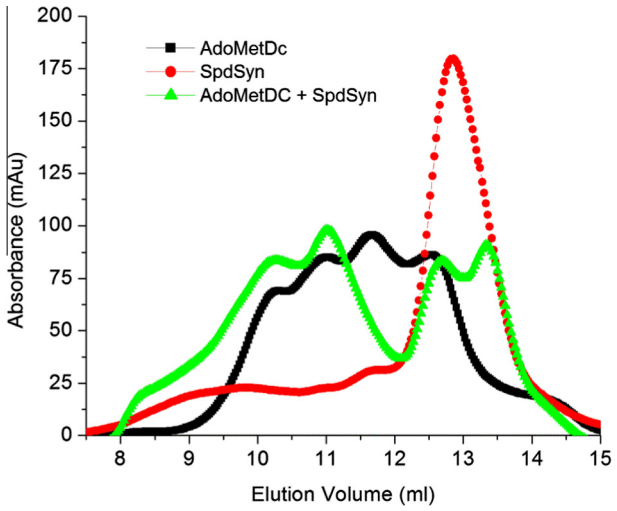

Fig. 4. Size exclusion chromatography of individual protein with SpdSyn (red), AdoMetDc (black) and their complex (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.2. Isothermal titration calorimetry

In order to confirm the interaction observed by GST-pull down assay, isothermal titration calorimetry experiments were performed to obtain the thermodynamic parameters of protein–protein interaction, the results of which are surmised in Fig. 3A, with the upper panel showing the raw data of 0.1 mM SpdSyn titrated into 0.01 mM AdoMetDc and the lower panel being the integrated plot for each injection after data fitting. Binding was exothermic, and was fitted using a "one set of sites" model to obtain the heat change (ΔH) of −93,800 kcal/mol, showing thermodynamically favorable interaction between *L. donovani* SpdSyn and AdoMetDc. The value for the dissociation constant (K_d) for the interaction was found to be 0.4 μM which suggests a moderate affinity between two proteins (Fig. 3A).

3.2.3. Fluorescence spectroscopic analysis of L. donovani SpdSyn–AdoMetDc interaction

The interaction were further analysed using fluorescence studies, using an external fluorophore, FITC. The fluorescence spectroscopic studies shows good agreement with ITC studies. The fluorescence emission maxima of FITC increases with increasing concentration of AdoMetDc. It shows that AdoMetDc interacts with SpdSyn showing the release of free FITC and interaction between two proteins (Fig. 3B and C). The control titration with Buffer alone has no significant change in fluorescence emission maxima. The apparent K_d value of interaction was 0.6 μM, comparable with the value obtained from titration calorimetry experiments, which further validates the proteins forming a complex formation. The Fluorescence spectroscopic study with labelled AdoMetDc and unlabelled SpdSyn also shows similar results (Data not shown).

3.2.4. Size exclusion chromatography

The oligomeric nature of *L. donovani* SpdSyn–AdoMetDc complex was obtained by purifying the complex using size exclusion chromatography. Individually, native SpdSyn elutes as a dimer (13.0 ml) on a S200 column while AdoMetDc exists in four oligomeric forms, with the tetramer being the most abundant (11.6 ml). When the incubated protein mixture is loaded on the same column, the pattern is distinctly different, as evident in Fig. 4. Two additional peaks corresponds to 12.6 ml and 13.6 ml appear, instead of the SpdSyn peak, while there is no peak corresponding to the tetrameric AdoMetDc. The additional peaks could be a hetero dimer which seems to suggest the possibility of the existence of a hetero-oligomeric species (Fig. 4). The exact stoichiometry is not clear by this analysis but seen in the context with other experimental results detailed above, further validate the possibility of complex formation.

These biophysical studies confirms that *L. donovani* SpdSyn and AdoMetDc interact with each other. These two proteins are present in all classes of organisms and have a conserved structural organization. Human SpdSyn–AdoMetDc are also supposed to interact on the basis of String database, with a comparatively lower score (0.951) than the corresponding *L. donovani* interaction (0.984), but higher than the *A. thaliana* SpdSyn-SpmSyn (0.924). As structures of human AdoMetDc and SpdSyn are available (PDB Ids 3EP9 and 3C6 K respectively), the two proteins were computationally docked using two different softwares, GRAMM X and ClusPro [26,27]. Both computational studies predict the complex formation, with ClusPro giving a balanced docking score of −799. As the individual proteins are structurally conserved across organisms, homology models of the corresponding *L. donovani* homologues were built using Modeller 9.13 [28]. *In silico* protein–protein docking studies suggest that these two do form a complex, with a higher balanced docking score (−1299). Analysis of the docked protein–protein complexes show that the interaction is primarily stabilized by hydrophobic and π–π stacking interactions (Supplementary Fig. 1). Further analysis of the interacting surfaces in the two complexes suggest that the *L. donovani* complex buries almost double the area as the human complex (10,000A^2 vs 5000A^2). These results suggest that the metabolon might be a better target for therapeutic studies, instead of the individual proteins. The functional and structural characterization of this interaction is further a matter of research and are in progress in the laboratory.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.008.

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