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Research Article

Identification of Possible Binding Sites on PfAdoMetDC by *E.coli* Trigger Factor Using Bioinformatics Approach

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Abstract

Plasmodium Falciparum S-Adenosylmethionine (PfAdoMetDC) plays an important role in the biosynthesis of polyamines. Contaminant proteins pose a major challenge in producing recombinant PfAdoMetDC protein. Some of the most distinct contaminants that co-purify with PfAdoMetDC are molecular chaperones. This study identified binding sites present in PfAdoMetDC that could make it amenable for recognition by some of the most distinct *E.coli* molecular chaperones, Trigger Factor (TF) and DnaK (Hsp70). Bioinformatics based predictive tools were used as the main approach to identify TF and DnaK binding sites on PfAdoMetDC. Well established *E.coli* TF interactors, 50S ribosomal subunit protein (L2) and protein chain Elongation Factor (ET Ts), together with *E.coli* AdoMetDC, were used as reference proteins which were then compared to PfAdoMetDC as a target protein. Out of the three *E.coli* proteins investigated, *E.coli* AdoMetDC possessed the highest score, followed by L2 protein with respect to hydrophobicity profiles. Based on these findings, PfAdoMetDC was found to be a good candidate for both TF or DnaK due to their interaction with exposed hydrophobic segments of client nascent peptides. The strongly hydrophobic character of PfAdoMetDC could suggest it co-purifies with *E.coli* chaperones following recombinant production in *E.coli*.

Keywords: Trigger Factor; DnaK; PfAdoMetDC; *Plasmodium falciparum*; *E.coli* AdoMetDC

Introduction

Malaria is still one of the leading global causes of deaths though the search for alternative drugs and vaccines for disease combating has gone for many years [1]. Malaria kills mostly young children (under the age of five) mostly from Africa. Of most particular concern, is the perpetual cycle of resistance to antimalarial in use? Therefore, the search for alternative vaccines and drugs for malaria is essential. The application of bioinformatics based tools to address biomedical challenges has revolutionized the pace at which structure-function features of molecules such as proteins could be deciphered [2]. However, protein function and structural properties may only be confidently inferred from biochemical and cell biological experimentation, whilst the design of inhibitors and evaluation of their interaction with the protein of interest are ultimately dependent on the availability of soluble and functional proteins. It is widely accepted that a specific protein of interest can only, under rare instances, be isolated in sufficient quantities from the natural host cell for downstream studies [3]. Otherwise the heterologous expression of the proteins is the most reliable approach to glean substantial yields of proteins for down-stream analysis. However, the overexpression of some recombinant proteins results in the formation of inclusion bodies [4-6]. The formation of these unwanted forms of products is usually not related to the size of the protein produced but rather tothe over-crowded environment prevailing in the expression host system. It has been suggested that even endogenous proteins may cause inclusion bodies if their production is too high in a cell [7].

Some proteins are produced in soluble forms or obtained in high quantities. However, yield is not always associated with quality as some highly produced recombinant proteins may exhibit poor activity [8]. In addition, some recombinant expressed proteins tend to co-purify with contaminant endogenous proteins [8]. It has been reported that PfAdoMetDC co-purifies with DnaK [9]. DnaK is one of the most prominent *E.coli* molecular chaperones. DnaK belongs to the heat shock protein 70 (Hsp70) family of molecular chaperones [10]. The contamination of recombinant PfAdoMetDC with *E.coli* DnaK is thought to be due to the multi-domain nature of PfAdoMetDC which necessitates a lengthy folding process during its production [11]. Molecular chaperones play an important role in the folding of the newly produced polypeptide upon its exit from ribosomes [12]. It is well known that for newly produced proteins require assistance during their folding stages, hence molecular chaperones facilitate this process [11]. Trigger factor (TF) is the first molecular chaperone to interact with non-native proteins, ultimately handing them over to DnaK for complete folding [12,13]. The cooperation of these two chaperones was reported as effective for the production of multi-domain proteins, such as β-galactosidase [13]. However, another possibility is that TF delays folding of multi-domain proteins [14-17]. Altogether, this could be explained why PfAdoMetDC may be a suitable substrate for TF and DnaK.

Kerner [18] showed that there are three classes of GroEL interacting substrates based on their chaperone dependence. Class I, included proteins that could spontaneously fold and whose refolding yield could be optimized by chaperone interaction. However, their solubility was independent of GroEL [19]. GroEL has an ability to bind exposed hydrophobic surfaces on substrates [20]. Hartl and

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Hayer-Hartl [21] demonstrated that trigger factor and DnaK/DnaJ/GrpE chaperones might interact with nascent polypeptides upstream of the chaperon in and most of the proteins exhibiting class I behavior had the opportunity to fold before reaching GroEL. Class II proteins are unable to fold spontaneously, therefore they solely depend on chaperones assistance for them to fold to their native structures [21]. The solubility of class II proteins has been suggested to be unaffected by overexpression of chaperones, meaning that their solubility status does not change [21]. Class III proteins on the other hand constituted proteins that were absolutely dependent on the assistance of GroEL both *in vitro* and *in vivo* for folding to their native state [21]. Such proteins were highly aggregation prone and were unable to fold spontaneously. It was suggested that DnaK was able to interact with class III substrates to suppress their aggregation but could not promote their folding. Such proteins were transferred downstream for subsequent folding by GroEL [21]. From these previous studies, it can be concluded that molecular chaperones are cooperative and certain substrates require more than one set of chaperone system to reach their native structures. In addition, the molecular size and other biophysical features of PfAdoMetDC strongly suggest that it is a suitable candidate for GroEL binding. Therefore, it was also important to assess whether PfAdoMetDC could benefit from these molecular chaperones for their synthesis and folding. In a recent study, it was confirmed that improved quality of PfAdoMetDC was produced in *E.coli* in the presence of supplemented plasmodial Hsp70 and *E.coli* GroEL [22].

PfAdoMetDC is involved in the formation of polyamines [23,24]. The polyamine synthesis pathway is a key antimalarial drug target [22]. However the production of PfAdoMetDC in *E.coli* is problematic since this protein always co-purifies with *E.coli* chaperone contaminants. The import of the current study was to establish the structural features of PfAdoMetDC that make it an attractive substrate of the *E.coli* molecular chaperones, TF and DnaK. Our findings suggest that PfAdoMetDC exhibits a high degree of hydrophobicity. Because of this quality, it is likely to be bound by *E.coli* molecular chaperones with high affinity. Our findings have implications on the current understanding in the field of recombinant protein biotechnology.

Experimental Design

Sequence alignment of Trigger factor interactors

The sequences of L2(P60422), L23 (POADZO); L29 (POA7M6), L3 (P60438); L13 (POAA10); S7 (PO2359) and EF-Ts (POA6P1) were obtained from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). All these substrates were picked due to their ability to interact with Trigger factor, hence were used as reference proteins [25]. Using Bioedit_v7. 0. 5. 3 software, sequence identity and similarity between PfAdoMetDC and *E.coli* AdoMetDC was determined following a method by Hall [26]. The sequence alignment was performed using Multiple ClustalW tool according to Thompson [27].

Hydrophobicity profiles and DnaK binding sites

The hydrophobicity profile of TF substrates (*E.coli* AdoMetDC) and PfAdoMetDC was predicted using a method by Kyte and Doolittle [28]. Hydropathy profiles were used to examine the surface features of proteins [24]. The peaks obtained on the positive side represented the hydrophobic profiles of the protein, while those on the negative side represented the hydrophilic profiles. DnaK/Hsp70 binding sites on *E.coli* TF substrates, *E.coli* AdoMetDC and PfAdoMetDC was predicted using a method by Rudiger and colleagues [29]. This method was based on differential scoring of the statistical energy contribution of each amino acid in a five residue core and four residue flanking regions, together constituting the proposed DnaK binding motif [30]. The combined energy value obtained for a given sequence was taken as a measure of the likelihood that DnaK binded to this sequence. Therefore, peptides that registered scores less than -5 were predicted as DnaK binders, while those that registered scores greater than -4 were regarded as non-binders.

Homology Modeling of *E.coli* TF substrates and PfAdoMetDC

Models of *E.coli* TF substrates, the *E.coli* AdoMetDC and PfAdoMetDC were generated using Modeller software package, version 8.2 [27] and the protein modeling server, SWISS-MODEL [28]. The models were subsequently visualized using PyMol, version 0. 99 rc6 to highlight the predicted binding sites of Hsp70 and TF.

The assessment of the structural features of *E.coli* substrates and PfAdoMetDC that determine their eligibility as a TF, DnaK or GroEL substrate

In *E.coli* , while the ribosome-associated trigger factor together with DnaK/Hsp70 system assist the de novo folding of at least 340 cytosolic proteins within a broad size range between 16 -167 kDa [12], GroEL chaperone machinery helps to fold 250–300 newly synthesized proteins, though preferring those with a molecular size ranging between 20 - 60 kDa [29]. In addition to that the hydrophobicity profile of the substrates is a major factor that promotes the interaction between GroEL and the substrate [30]. Therefore, the analysis of both *E.coli* substrates and PfAdoMetDC features was compared to the previously published data [30-32]. The physicochemical features of typical GroEL substrate have been extensively analyzed [32]. Based on the previous studies, Isoelectric Point (pI), pH, hydrophobicity profile and sizes are crucial determinants peptides as GroEL substrates. Using these guidelines, the physicochemical features of *E.coli* susbtrates and PfAdoMetDC were analyzed to confirm their eligibility as GroEL substrates.

Results

In order to establish proteins that are predicted to interact with TF chaperone, String software (version 10.0) was used [33], and the results obtained are presented in Figure 1. Proteins that showed high hydrophobicity were preferred for comparison purpose, in order to observe the domains that interact with TF or if they share any similarities. Sequence alignment did not show any similarities between these proteins, suggesting that TF could be recognizing not only as hydrophobic proteins but hydrophilic proteins as well [17]. *E.coli* L2 and EF-Ts, with 0. 994% and 0.996% scores respectively, were used throughout this study as reference proteins.

Firstly, a protein sequence alignment of *E.coli* AdoMetDC and PfAdoMetDC was conducted in order to identify conserved TF and DnaK binding motifs (Figure 2). We assumed that *E.coli* AdoMetDC would-be well recognized by *E.coli* DnaK and TF. Secondly, we

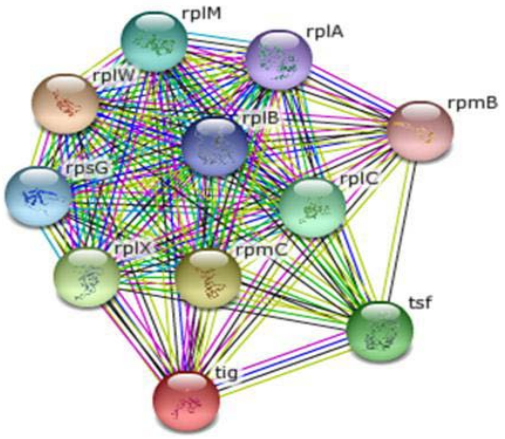

Figure 1: Protein–protein interaction network visualized by STRING. In this view, the color saturation of the edges represents the confidence score of a functional association between Trigger Factor and *E. coli* substrates. Abbreviations of the substrates of Trigger Facto: rplM, 50S ribosomal protein L13; rplA, 50S ribosomal protein L28; rpmB, 50S ribosomal protein L1; prlC, Oligopeptidase; rplW, 50S ribosomal protein L23 rpmC, 50S ribosomal L29 prlX, 50S ribosomal protein L31 and tig, Trigger Factor.

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                    110       120       130       140       150
                    ....|....|....|....|....|....|....|....|....|...
PfAdoMetDC          DLLIYHMDNVGIIEKNCVYDETFIENEKFHNIAEFIKEHFLYCFFTHMNYRNK
E. coli AdoMetDC    KLKLHGFNNLTKSLSFCIYDICYAKT----------------------AE
Clustal Consensus   .* :: ::*:    . *:**  : :.                       :

                    210       220       230       240       250
                    ....|....|....|....|....|....|....|....|....|...
PfAdoMetDC          SSDDVHMTDIASTFKFCSEIHLFGINKYNEKNQFHDAYLNNKSLNLFTRVHED
E. coli AdoMetDC    ------LTEILS-----ETCSIIGANILNIARQDYEP--QGASVTILVSEEPV
Clustal Consensus         :*:* *      .  ::* *  *  .* ::.  :. *:.::.  .

                    310       320       330       340       350
                    ....|....|....|....|....|....|....|....|....|...
PfAdoMetDC          NNIENIPSIENKESNNNSRCCHNNNYSGSCHNIVSVVPSERNNDHVHHRHYED
E. coli AdoMetDC    TYPESHP--------EGGLCTFRADIEVSTCGVISPLKALNYLIHQLESDIVT
Clustal Consensus   .  *. *        :., * .. : . *  .::* : : .   *  . .
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Figure 2: Sequence alignment of PfAdoMetDC and *E. coli* AdoMetDC. ClustalW alignments for PfAdoMetDC (E. C. 4. 1. 1. 50) and *E. coli* AdoMetDC (A7ZW69. 1) were performed using Bioedit programme ClustalW alignment. Charged polar and hydrophobic amino acids are highlighted by black box that is thought to be playing a critical role in protein folding). * denotes identical amino acids; " a conserved amino acids while"denotes semi-conserved amino acids.

assumed that certain conserved amino acid residues that constitute motifs for DnaK and TF recognition occur in PfAdoMetDC. These amino acids are highlighted by black boxes in Figure 2. The sequence of *E.coli* AdoMetDC served as a reference point for the assessment of PfAdoMetDC for both TF and DnaK substrate. PfAdoMetDC shared a sequence identity of 33% and similarity of 50. 09% with *E.coli* AdoMetDC, which suggested that they were closely related homologs (Figure 2). Altogether, this indicated that PfAdoMetDC, though not from the same species with *E.coli* AdoMetDC, could be recognized by DnaK/Hsp70 and TF chaperones. The hydrophobicity profiles of *E.coli* TF substrates (L2 protein (55 kDa), EF-Ts protein (48 kDa) and *E.coli* AdoMetDC (41 kDa) were used as controls to investigate the suitability of PfAdoMetDC (60 kDa) as a substrate for TF and DnaK chaperones. It is important to note that *E.coli* AdoMetDC has a smaller molecular size than PfAdoMetDC, thus the Y-axis (score) values were different from PfAdoMetDC values (Figure 3). As was expected, *E.coli* AdoMetDC showed highest scores of hydrophobicity

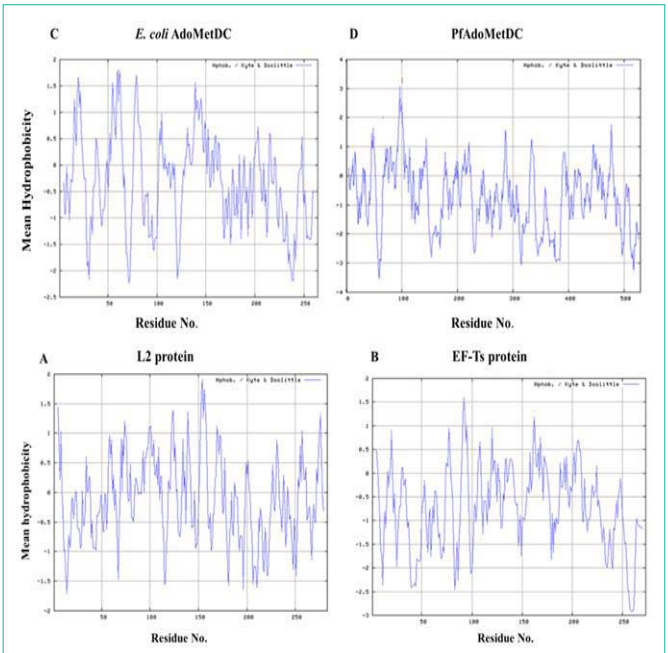

Figure 3: Hydrophobicity profiles of the two of *E. coli* AdoMetDC and PfAdoMetDC substrates. A and B are hydrophobicity profile analysis of L2 and ET-Ts substrates, indicating the abundance of hydrophobic (negative scores) compared to hydrophilic profile (positive scores) amino acids residues. C and D are *E. coli* AdoMetDC and PfAdoMetDC, indicating the abundance of hydrophilic (negative scores) compared to hydrophobic profile (positive scores, denoted with stars) amino acids residues.

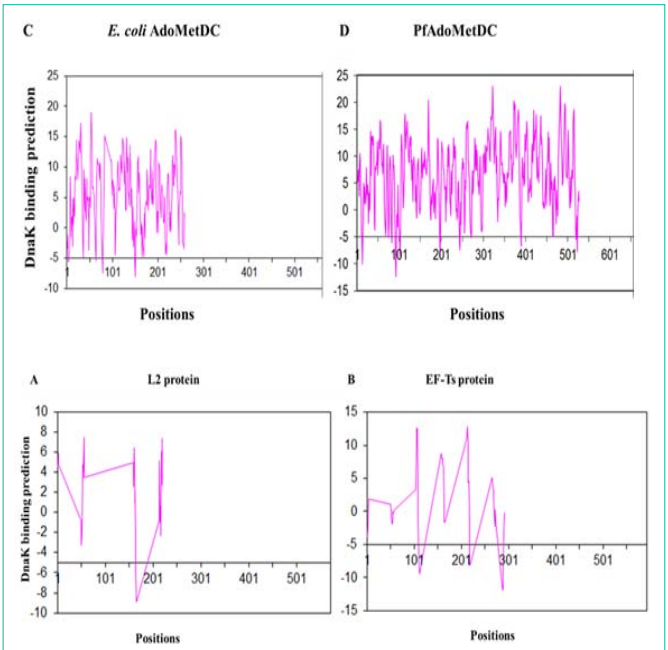

Figure 4: Prediction of DnaK binding on *E. coli* AdoMetDC and PfAdoMetDC substrates. (A) Prediction of DnaK binding on L2 protein, (B) ET-Ts to DnaK prediction binding, (C) and (D) are prediction of potential DnaK binding sites on *E. coli* AdoMetDC and PfAdoMetDC proteins respectively. Segments with less than -5 indicate potential DnaK binding sites within PfAdoMetDC protein sequence.

profiles when compared to PfAdoMetDC (Figures 3A and 3B). However, there were residues that registered higher scores on

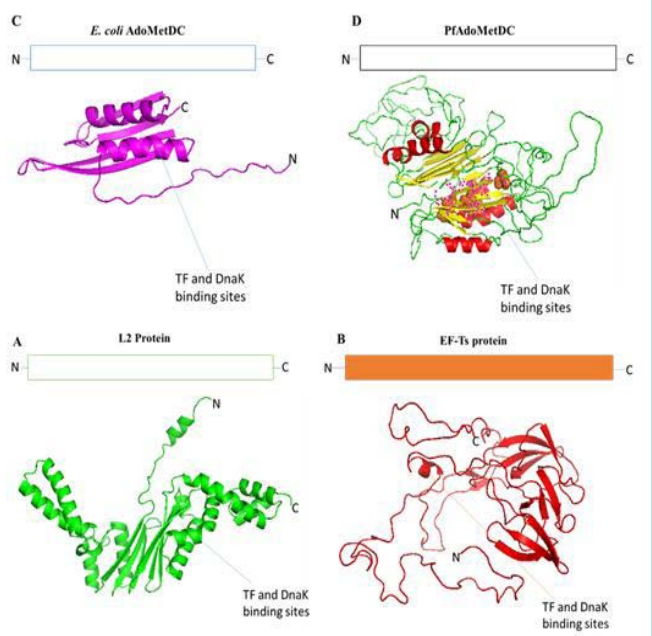

Figure 5: Modeling of ET-Ts and L2 TF substrates. (A) ET-Ts linear structure and its model protein, (B) L2 linear structure and its model protein (C) *E. coli* AdoMetDC protein and (D) PfAdoMetDC protein with its linear structure.

PfAdoMetDC at position 1-101 on the β-sheet (Figure 3).

Figures 4A and 4B show the binding scores of DnaK on *E.coli* L2 and EF-Ts TF interactors, while Figures 4D and 4C indicates *E.coli* AdoMetDC being compared to PfAdoMetDC protein sequences. The results showed that L2 and ET-Ts proteins are not likely to interact with DnaK, and thus did not correspond to the hydrophobicity scores (Figure 4A and 4B). On the other hand, DnaK displayed high binding affinity on PfAdoMetDC when compared to *E.coli* AdoMetDC. Size differences of these two proteins could be a main contributing factor. Thus, the PfAdoMetDC profiles suggested that they were suitable candidates or substrates for DnaK. Based on these findings, it could therefore be concluded that PfAdoMetDC possessed more hydrophobicity residues. From a study by Hartl and Hayer-Hartl [13] it was obtained that smaller proteins do not need more assistance during their folding. However, larger proteins interacted with molecular chaperones such as DnaK and GroEL in order to stabilize them during their folding process.

A 3D model of L2, ET-Ts, *E.coli* AdoMetDC and PfAdoMetDC was generated. The generated TF were not full length proteins. *E.coli* AdoMetDC had α-helix at the N-terminal and β-sheet at the C-terminal (Figure 5C). In contrast PfAdoMetDC had a β-sheet at the N-terminal and α-helix at C-terminal (Figure 5D).

Based on the analysis of the predicted 3D structures of the proteins, hydrophobic residues were more pronounced at the α-helix compared to the β-sheet of the protein, thus the proposed binding sites of Hsp70s were at the N-terminal residues between 20-149 amino acids [26]. Further analysis also showed that PfAdoMetDC structure had a long loop, which contributed to the flexibility of the protein (Figure 5D) [15]. It is believed that these loops were prone to proteolytic attack if the protein were not properly folded [34]. Based on the hydrophobicity profiles of PfAdoMetDC, the protein has more hydrophobic regions in the β-sheet at residues 10-81 [26]. The proposed sites at which TF and DnaK bind during its folding are presented in Figures 5C and5D.

Discussion

The role of molecular chaperones is to bind to hydrophobic patches of the substrate until it is properly folded [31]. On the other hand, suitable client proteins for interaction with molecular chaperones are proteins with exposed hydrophobic regions. Even though *E.coli* AdoMetDC and PfAdoMetDC are from different species, based on the sequence alignment, results indicated that they shared hydrophobic amino acids (such as leucine and glycine) that could be recognized by TF and DnaK chaperones [35]. TF and Hsp70s bind to proteins approximately 20-60 kDa, preferably with exposed hydrophobic patches [31,36]. *E.coli* AdoMetDC was predicted to be 30 kDa and PfAdoMetDC was found to be 60kDa (Figure 4). Based on the analysis, *E.coli* AdoMetDC had pI value of 6. 0 while PfAdoMetDC had 6. 35. Mehlin and colleagues [3] suggested that malarial proteins that were difficult to express in *E.coli* were due to the fact that they had a pI above 6. The effect of the protein size on recombinant protein production in *E.coli* system is well known [16]. Size variation between TF and Hsp70 suggests that they both prefer binding to large proteins. This gives suggests that PfAdoMetDC might have more binding motifs that could be recognized by TF and Hsp70 chaperones, compared to *E.coli* AdoMetDC protein (30 kDa) (Figure 5D and 5C).

Kyte and Doolittle analysis demonstrated that *E.coli* AdoMetDC had more pronounced hydrophobic profiles than PfAdoMetDC, suggesting that the protein was a good candidate for recognition by molecular chaperones (Figure 3C and 3D). Williams and colleagues [9] suggested that PfAdoMetDC protein could be expressed in *E.coli* BL21 StarTM cells, however the major challenge was that the PfAdoMetDC co-purified with a contaminant, a 70 kDa*E.coli* DnaK. Hydrophobicity likely seems to be the main driving force for Hsp70 to interact with newly synthesized proteins. The exposure of hydrophobic regions by PfAdoMetDC was shown to result in the increased recruitment of DnaK/Hsp70 (Figure 5).

DnaK binding sites on PfAdoMetDC were predicted to be more frequent compared to *E.coli* AdoMetDC based on the predictive tool is that determines the possible binding motifs of DnaK on substrates (Figure 5) [28]. The obtained results indicated that DnaK binds to the β-sheet of the protein with high pronounced hydrophobicity residues. On the other hand, DnaK binds to the α-helix on *E.coli* AdoMetDC (Figure 5). DnaK is known to interact with both side chains and peptide backbone, while DnaJ only binds to side chains, to ensure that these chaperones interact with hydrophobic sequences without strong preference on the sequence [26].

It has been suggested that for chaperones to distinguish between folded and misfolded substrates, hydrophobic sequences are normally found on the interior of the folded substrates [31]. Together, these features appeared to have provided the necessary promiscuity that permitted them to bind a wide range of substrates [37]. A proteomic study using a temperature sensitive GroEL mutant strain, demonstrated that DnaK and DnaJ bound all the GroEL

substrates. These findings demonstrated that the GroEL-dependent proteins also contained predicted DnaK-DnaJ binding sites [37], an indication that like DnaK-DnaJ-GroEL system could possibly interact with a relatively large number of protein substrates including PfAdoMetDC protein. This suggests that the interaction of *E.coli* DnaK could be unbeneficial for the fold of PfAdoMetDC. On the other hand, supplementation of *E.coli* cells with plasmodial Hsp70s and GroEL seemed to favor folding of PfAdoMetDC. This suggests that in spite of their conservation Hsp70 proteins exhibit distinct functional specificity.

Conclusion

The focus of the current study was on analysis of the preferable binding sites by TF on PfAdoMetDC protein. We employed the DnaK binding predictive tool [12] was used because both DnaK and TF recognize hydrophobic patches on the substrate. Our results show that PfAdoMetDC can interact with both chaperones based on its hydrophocity profile. In addition, the fact that TF binds a wide range of nascent proteins could imply that PfAdoMetDC may also be recognized by this chaperone. However, it is possible that binding of PfAdoMetDC by TF may delay its folding. Ultimately, this leads to the stable binding of PfAdoMetDC by DnaK.

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