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In Vitro Refolding of Triosephosphate Isomerase from *L. donovani*

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Abstract The triosephosphate isomerase of *Leishmania donovani* (LdTIM) was expressed at high level in *Escherichia coli*. The TIM gene was cloned in expression vector pET-23(a) with C-terminal 6× His tag fused in frame, and expressed as a 27.6-kDa protein in *E. coli* as inclusion bodies. The recombinant LdTIM from *E. coli* lysate was solubilized in 6 M guanidine hydrochloride and purified by Ni-NTA chromatography. In the present study, the effect of bovine serum albumin on the reactivation of TIM was investigated. Furthermore, 8-anilino-1-naphthalene sulfonic acid was used to detect the structural changes induced by bovine serum albumin (BSA). Here, we conclude that BSA assists in the refolding and regain of LdTIM enzyme activity by providing framework for structure formation. This study indicates that numerous protein–protein contacts are constantly occurring inside the cell that leads to the formation of native protein.

Keywords *Leishmania donovani* · Triosephosphate isomerase · Guanidine hydrochloride · Isopropyl β-D-thiogalactopyranoside · 8-Anilino-1-naphthalene sulfonic acid

Introduction

The last step in protein biosynthesis is the folding of newly formed protein. Chain folding in vivo is completed within, at the most, a few minutes, and presumably gives exclusively active, functional protein, whereas chain folding in vitro, on the other hand, is often slow and seldom leads exclusively to active product; thus, there may be important differences in the routes of folding. There is very little known about either process to be sure of this, and so, there is much current work on the refolding of proteins. Single-subunit proteins lend themselves to studies on unfolding and refolding; experiments on the kinetics in both directions are especially helpful in selecting mechanisms [1–3]. Multisubunit proteins (oligomers) are less easy to study, but have additional features of interest. The kinetics of unfolding and refolding of oligomers may be difficult to study in both directions [4]. In

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Kinetoplastida, the seven enzyme of glycolytic pathway converting glucose to 3-phosphoglycerate are localized in glycosomes, whereas in others, these are cytosolic. The unusual localization of these enzymes which supposedly endows them with specific structural features, and the crucial importance of glycolysis for the parasites energy supply, makes the glycosome and its constituting proteins ideal targets for specific drugs [5]. Triosephosphate isomerase (TIM) is an enzyme of glycolytic pathway which interconverts glyceraldehyde-3-phosphate to dihydroxyacetone phosphate. Triosephosphate isomerase is TIM-barrel protein. TIM barrels are considered α/β protein folds because they include an alternating pattern of α-helices and β-strands in a single domain. Several TIM-barrel proteins are extensively studied in terms of folding and reactivation [6, 7]. There are intrinsic factors in cells that lead to the formation of native proteins, and numerous protein–protein contacts are constantly occurring. On these grounds, we explored if LdTIM protein has the capacity to interact with apparently unrelated proteins. There are several reports in which the reactivation of various enzymes has been measured in presence of bovine serum albumin [8–12]. The present paper describes studies on refolding and the regain of enzyme activity of triosephosphate isomerase in presence of bovine serum albumin.

Material and Methods

Chemicals and Reagents

Escherichia coli host strain BL21 (DE3) and the plasmid vector pET-23(a) were purchased from Novagen, UK. Restriction enzymes used for cloning were obtained from MBI fermentas. pGEMT easy vector and Ni-NTA superflow were obtained from Qiagen. Glyceraldehyde-3-phosphate, NADH, alpha-glycerophosphate dehydrogenase, and bovine serum albumin were purchased from Sigma. All other biochemicals were of the highest grade available.

Parasite Culture

The WHO reference strain of *Leishmania donovani* (MHOM/IN/80/Dd8) was obtained from Imperial College London (UK) and maintained in vitro as promastigotes in high glucose RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum containing 40 μg/ml gentamycin at 25 °C [13].

Primer Design and PCR Amplification of the Gene

PCR primers 5′-GAATTCATGTCCGCCAAGCCCCAGCCG-3′ (forward) and 5′-AAGCTTCCGGGTGGCATCAATGATGTCTCG-3′ (reverse) with Eco RI and Hind III restriction sites shown as underlined were designed on the basis of *Leishmania mexicana* TIM gene sequence available from NCBI nucleotide database (Accession No. P48499). Amplification of TIM gene was carried out using genomic DNA of *L. donovani* as template using the following reaction cycles: 1 cycle for 4 min at 94 °C, 30 cycles for 45 s at 94 °C, 45 s at 54 °C, 2 min at 72 °C, and 1 cycle for 10 min at 72 °C.

Cloning in pGEM-T Easy Vector

The pGEM-T Easy vector (Promega) was used to clone the complete open reading frame (ORF) of TIM from *L. donovani*. The resulting PCR product and pGEM-T Easy vector

were ligated at 4 °C overnight using T4 DNA ligase to obtain recombinant construct. This construct was transformed into *E. coli* DH5α cells, and the resulting colonies were screened by restriction digestion analysis. Nucleotide sequencing of positive clones were carried out from both the directions and sequence submitted to gene bank.

Multiple Sequence Alignment

TIM gene of diverse species at the level of deduced amino acid sequence from Swiss-Prot (http://www.expasy.org/sprot) was aligned with Clustal W (www.ebi.ac.uk/clustalw).

Construction of Expression Vector

The TIM gene from pGEM-T construct was digested with EcoRI and Hind III restriction endonucleases and subcloned in pET-23(a) vector digested with same enzymes to produce the recombinant plasmid that contains C-terminal His_6-tag for purification purpose. The clones were screened and confirmed by single digestion of plasmids with restriction enzyme Hind III. The positive clone containing recombinant construct was taken for expression and purification.

Expression and Purification under Denaturing Conditions

The recombinant construct was used to transform competent *E. coli* BL21 (DE3). The plasmid was grown overnight as primary culture. Luria–Bertani broth (100 ml) containing 100 μg/ml ampicillin was reinoculated at 0.1% cell density and grown at 37 °C under constant shaking. The culture was induced by 0.1 mM isopropyl-β- D-thiogalactopyranoside (IPTG; $OD_{600\ nm}$ 0.4) and incubated at 20 °C for another 12 h under gentle shaking at 120 rpm. The overnight grown culture was harvested at 5,000 g for 15 min at 4 °C and suspended in 10 ml buffer A (100 mM triethanolamine buffer, 10 mM EDTA, 6 M guanidine hydrochloride, pH 8.0). The cell suspension was sonicated (8×30 s, Sonicator Heat system with 50% duty cycle) on ice in the presence of protease inhibitor PMSF. The resulting cell lysate was centrifuged at 14,000 rpm for 30 min. The supernatant containing recombinant protein was loaded on to the column packed with 1 ml Ni-NTA superflow. The column was washed with 50 ml of buffer B (100 mM triethanolamine buffer, 10 mM EDTA, 6 M guanidine hydrochloride, pH 6.3) followed by 50 ml of buffer C (100 mM triethanolamine buffer, 10 mM EDTA, 6 M guanidine hydrochloride, pH 5.9). The protein was eluted with elution buffer D (100 mM triethanolamine buffer, 10 mM EDTA, 6 M guanidine hydrochloride, pH 4.5). The eluted fractions were collected and stored at 4 °C. Fractions were analyzed by 12% SDS–PAGE [14].

Enzyme Activity Assay

Activity in the direction of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate was determined at 25 °C as described elsewhere [15]. Activity was followed by the decrease in absorbance at 340 nm as a function of time in an evolution 300 spectrophotometer (Thermo). One unit (U) of enzyme is defined as the conversion of 1 μmol substrate/min at 25 °C. Specific activity was expressed as units/mg protein.

Reactivation of Enzyme

Reactivation of the purified denatured LdTIM was monitored at different concentration of denatured LdTIM (1–20 μg/ml) in media at pH 7.4 that contained 100 mM triethanolamine,

10 mM EDTA, and 200 mM GdnHCl in presence and absence of (10 μg/ml) BSA, incubated at 25 °C. The activity was measured at various time intervals (0.0, 0.5, 1, 2, 3, 5, 8, 20, and 24 h). The titration curve of different concentrations of (0–80 μg/ml) BSA on LdTIM (10 μg/ml) reactivation was also performed as described above.

8-Anilino-1-Naphthalene Sulfonic Acid Binding

Fluorescence experiments were performed with Spectramax M2 spectrofluorometer (Molecular devices). The buffer used for measurements was 100 mM triethanolamine buffer (pH 7.6). The concentration of LdTIM in the assay was 10 μg/ml in the absence and presence of 10 μg/ml BSA incubated at 25 °C for 5 h. Fluorescence of 8-anilino-1-naphthalene sulfonic acid (ANS) was excited at 360 nm, and emission spectra were recorded between 400 and 600 nm. For data evaluation, ANS (100 μM) emission spectra in buffer were subtracted from the corresponding ANS/protein spectra, thereby giving the relative fluorescence intensities (RFU) as a function of the wavelength.

Results

Cloning, Overexpression, and Purification

An open reading frame of 753 base pairs encoding 251 amino acids was amplified from *L. donovani* genomic DNA (Fig. 1a). Authenticity of the gene was confirmed by DNA sequencing. The nucleotide sequence of LdTIM was submitted in GenBank under accession no. DQ649411. LdTIM showed 49.2%, 46.3%, 66.6%, and 88.10% identity with human, mouse, *Trypanosoma cruzi*, and *L. mexicana* respectively. The motif in LdTIM AYEPVWAIGTG (166–176) has been conserved between other trypansomatids. LdTIM has cysteine residue at position 15, while the corresponding residue in human is methionine. The parasites TIM gene having the cysteine residue at this position had been targeted to achieve species-specific inhibition in *Trypanosoma brucei*, *T. cruzi*, and *L.*

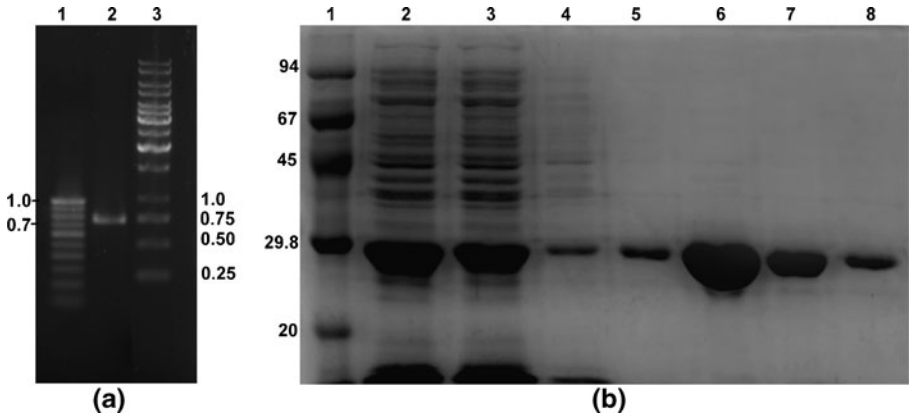

Fig. 1 **a** PCR amplification of TIM gene. *Lanes 1* 100 bp ladder, *2* amplified product of 753 bp, *3* 1 Kb DNA. **b** SDS–PAGE analysis of cellular fractions and purified protein. *Lanes 1* low molecular protein marker, *2* induced BL21(DE3) cells containing pET-23(a)-LdTIM, *3* pellet fraction of cells containing pET-23(a)-LdTIM, *4* fraction of wash buffer pH 6.3, *5* fraction of wash buffer pH 5.9, *6–8* elutes collected in pH 4.5 buffer

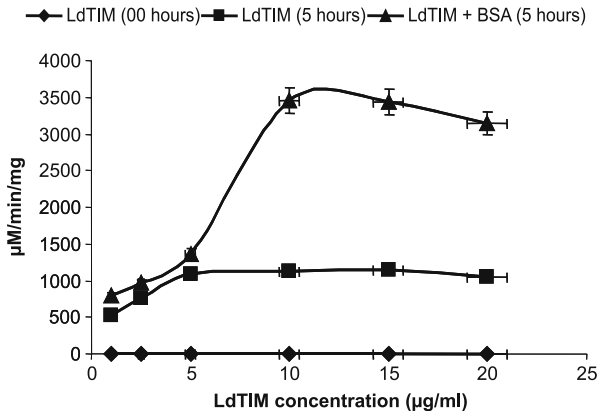

Fig. 2 Reactivation of LdTIM at various protein concentrations in the presence and absence of 10 μg/ml BSA after 5 h of incubation in reactivation media

mexicana [16]. The LdTIM was expressed into pET-23a (+) expression vector under the control of a strong bacteriophage T7 promoter. The correct orientation of the clone was confirmed by restriction digestion analysis. The expressed protein was found in inclusion bodies. The recombinant TIM protein present as inclusion bodies was solubilized in 6 M guanidine hydrochloride and further purified by using Ni-NTA affinity chromatography. Molecular weight of the protein encoded by the LdTIM, as estimated by SDS–PAGE, is approximately 27.6 kDa (Fig. 1b). The yield of purified protein obtained from 100-ml culture was 2 mg.

Reactivation of LdTIM

Denatured LdTIM was allowed to reactivate at protein concentrations that range from 1 to 20 μg/ml in the presence and absence of 10 μg/ml BSA in the reactivation media. The activity was measured at various time intervals as described in "Material and Methods" section. The maximum reactivation of denatured protein, i.e., 3.4×10^3 μM/min/mg was observed at 5 h with 10 μg/ml LdTIM concentration with BSA in reactivation buffer while

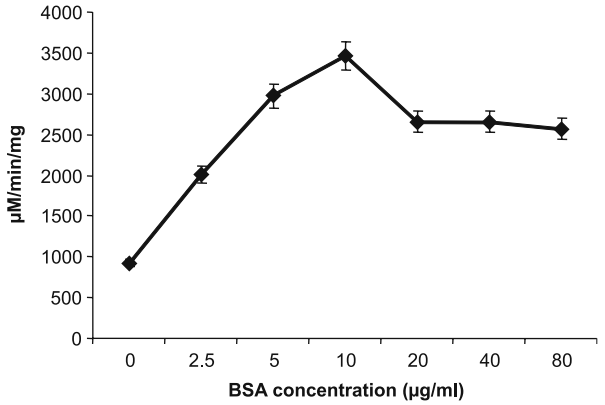

Fig. 3 The titration curve of different concentrations of BSA on LdTIM reactivation after 5 h of incubation in reactivation media

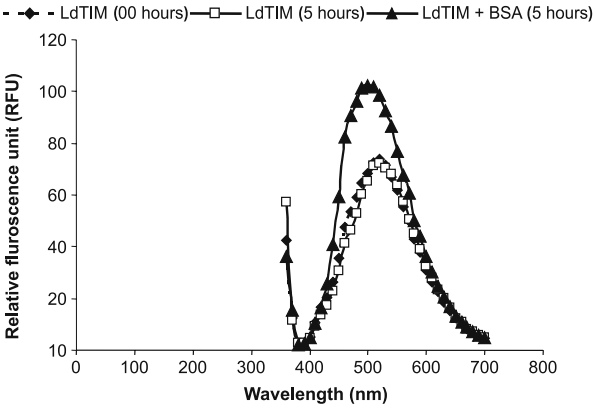

Fig. 4 The fluorescence spectra of ANS in presence of LdTIM with or without BSA. Plot of relative fluorescence unit (*RFU*) vs. wavelength

1.1×10^3 μM/min/mg activity at same LdTIM concentration without BSA in reactivation buffer. After 5 h, the reactivation had remained constant for 24 h. This result indicates that the maximum extent of reactivation of denatured protein was attained in 5 h. The effect on LdTIM activity with increasing concentration of LdTIM at time zero is also shown (Fig. 2). The titration curve of different concentrations of BSA (0–80 μg/ml) on reactivation of LdTIM at 10 μg/ml showed that, the maximal effect of BSA was attained at concentration of 10 μg/ml in 5 h. The favorable action of BSA is slightly diminished when it was added at concentration above 10 μg/ml (Fig. 3). In the absence of BSA, and in consonance with other works [17–19], we observed that the extent of reactivation increased with protein concentration indicating that the rate of reaction 2 is central in the reactivation of LdTIM.

Structural Changes in LdTIM Induced by BSA

The dye ANS has been used as a probe for the detection of structural changes. The fluorescence emission spectrum of ANS was same for LdTIM at time zero as well as at 5 h in absence of BSA, while the maximum of the emission curve was shifted toward shorter wavelength, i.e., 510 to 490 nm with increase in fluorescence in LdTIM samples incubated for 5 h in presence of BSA, suggesting that BSA induces the structural changes in denatured LdTIM (Fig. 4).

Discussion

Expression of cloned genes in bacteria is widely used both in industry, for the production of pharmaceutical proteins, and in research, for the production of proteins for structural and/or biochemical studies. Bacteria produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes; however, the product of interest is frequently deposited in insoluble inactive aggregates or inclusion bodies. The general strategy used to recover active protein from inclusion bodies involves three steps: firstly, inclusion body isolation and washing; secondly, solubilization of the aggregated protein, which causes denaturation; and finally, refolding of the solubilized protein. While the efficiency of the first two steps can be relatively high, folding yields may be limited by the production of

inactive misfolded species as well as aggregates. Formation of off-pathway species, such as incorrectly folded species and aggregates, are the cause of decreased renaturation yields. Because aggregation is an intermolecular phenomenon, it is highly protein concentration-dependent. The most direct means of minimizing aggregation is by decreasing protein concentration. It has been suggested that optimum recovery yields can be expected if the protein concentration is in the range of 10–50 μg/ml [20]. The manner in which a newly synthesized chain of amino acids transforms itself into a perfectly folded protein depends on both the intrinsic properties of the amino acid sequence and multiple contributing influences from the crowded cellular milieu [21]. Protein molecules, however, all have a finite tendency either to misfold or to fail to maintain their correctly folded states under some circumstances [22]. Thus, correct folding and misfolding are a competing process during protein folding in intracellular environments. As shown in a study [23], an intrinsically disordered protein FlgM gains structure in living *E. coli* cells and under physiologically relevant conditions in vitro, which suggests a reason for the observation that some proteins are only folded under physiologically relevant conditions and proves the biological relevance of studying proteins in vivo and at physiologically relevant solute concentrations. However, some proteins do not become structured in vitro at physiologically relevant solute concentrations, which may require another protein to provide a framework for structure formation. For example, certain transcription factors remain disordered even in solutions containing high concentrations of macromolecular crowding agents, yet they gain structure in the presence of other components of the complex [24]. It should be pointed out that in vitro simulations of macromolecular crowding in vivo do not necessarily provide results that are more meaningful in a biological context, although they may be appropriate in certain specific experiments. Significant nonspecific interaction is an unavoidable consequence of crowding and confinement in most or all physiological fluid media [25]. Our data suggest that BSA induces the structural changes in denatured LdTIM, which resulted in refolding and regain of LdTIM enzyme activity by providing framework for structure formation. Similar data were obtained with TbTIM [26], indicating that TIM dimers have the capacity to interact with a wide variety of proteins. This would be in consonance with reports that show that TIM interacts with actin, microtubules, and membranes [27–30] and that TIM binding to the later structures is affected in human TIM deficiencies [29]. Likewise, it has been shown [31] that TIM, as well as other glycolytic enzymes, interacts with the K_{ATP} channel of the plasma membrane and thereby regulates its function. Thus, the collective data indicate that LdTIM is promiscuous that it interacts with several unrelated proteins in intracellular compartment to get its native form.

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