Chapter 7

Entamoeba histolytica PGDH and PSAT: Characterization of novel protein-protein interactions
7.1 Introduction

Amino acids, in addition to their role as protein monomeric units, are energy metabolites and precursors of many biologically important nitrogen containing compounds. The amino acid metabolic pathways have considerably evolved during the course of evolution (Ali et al. 2004a). Protists are the most simplified unicellular organisms and majority of them lack certain metabolic pathways viz amino acid metabolism. However, parasitic protists are generally known to possess a simplified amino acid metabolism (Ali et al. 2004a; Ali and Nozaki 2006). They have limited set of enzymes involved in amino acid synthesis. Over the past decade serine and its related metabolism is been extensively explored in Entamoeba histolytica which is one of the two exceptions of the unicellular eukaryotes that possess such a metabolism, the other one is Leishmania (Ali et al. 2004a). It is now an established fact that this primitive, unicellular, pathogenic and amitochondriate protist adopted genes for such a metabolism through lateral gene transfer form bacteroides over the course of evolution (Ali et al. 2004a; Ali et al. 2003).

The first step in the anabolism of the serine is reversible oxidation-reduction reaction catalyzed by D-3-phosphoglycerate dehydrogenase where 3-PGA is converted into phosphohydroxy pyruvate (HPAP). The second step involves conversion of HPAP into phosphoserine (PS) in a reversible transamination reaction catalyzed by phosphoserine aminotransferase (PSAT). Subsequently PS is processed into serine or cysteine by phosphoserine phosphatase (PSP) or cysteine synthase (CS), respectively (Westrop et al. 2006).

It's a well established fact that proteins carry out their cellular functions through their concerted interactions with other proteins. Such protein-protein interactions can be classified as permanent or non-obligatory depending on the ability of the proteins to exist independently (Tompa et al. 1987). Metabolic pathways are often orchestrated by sequential enzymes which operate in perfect synchrony by interacting with each other in the form of highly organized complexes. Such molecular complexes provide highly efficient, regulated and targeted metabolite flux through a pathway (Huang et al. 2001). These supramolecular complexes have been extensively characterized in the mitochondrion, especially enzymes of the TCA cycle (Hearl and Churchich 1984).

Entamoeba histolytica possess a unique D-phosphoglycerate dehydrogenase (EhPGDH) which is a homodimer of approximately 70 kDa with only two domains the substrate binding domain (Sbd) and the nucleotide binding domain (Nbd). The active site cleft is present at the interface of the two domains. EhPGDH lacks the Trp139 residue which is considered to be crucial for the tetramerization of the protein (Ali et al. 2004a). Most interestingly the protein is insensitive to feedback inhibition
from L-serine because of the absence of a regulatory domain, a complete contrasting feature from its counterparts in *E coli* and *Mycobacterium tuberculosis* (Ali et al. 2004a; Dey et al. 2005b).

In the present study we have investigated unique structural features of EhPGDH especially novel interactions between EhPGDH and *Entamoeba histolytica* phosphoserine aminotransferase (EhPSAT), the first two enzymes of the phosphorylated serine biosynthesis pathway using standard *in vitro* protein-protein interaction techniques. Furthermore, for understanding the role of the two domains of EhPGDH in such protein-protein interactions as-well-as stability of the protein, separate studies on the isolated nucleotide binding domain (Nbd) were also carried out. Our results provide a possible evidence for substrate channelling between EhPGDH and EhPSAT in this novel metabolon.

7.2 Materials and Methods

**Materials**

Same as previously described in chapter 3.

**Methods**

*Overproduction and purification of EhPGDH AND EhPSAT:* The proteins, EhPGDH-His₆ and EhPSAT-His₆ were over-expressed and purified by method described earlier in chapters 3 and 6 ( ).

*Cloning, over-expression and purification of GST-EhPGDH:* EhPGDH encoded by an 897 base pair (bp) gene fragment was PCR amplified from the full length gene of EhPGDH (pET-15b-EhPGDH). The PCR was performed with primers (forward-5'GGATCCAGATAGTTGTGATAACCGA-3' and reverse-5'CTCGAGTTAGAACCTATTGACTTGGAA-3') with BamHI and Xhol restriction sites. PCR conditions used were: 1 X 94 °C for 5 minutes, 30 X 94 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 1 minute and finally 1X 72 °C for 10 minutes. The amplified fragment was cloned in pGEX-4T1 (GE healthcare life sciences) vector between BamHI and Xhol restriction sites in the same orientation as the LacZ promoter to produce GST-EhPGDH. The resultant constructs were transformed into *E coli* BL-21 competent cells for expression. A single colony of BL-21 LB ampicillin plate was inoculated into 5 mL of LB broth (HI media) having ampicillin at a concentration of 100 µg/mL and allowed to grow overnight at 37 °C. It was then sub cultured in a 500 mL LB broth at 37 °C until A₆₀₀ of 0.6 was achieved. The culture was then induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside and was further incubated at 20 °C for 16 hours. The cells were harvested at 8000 rpm for 10 minutes and the resultant pellet was suspended in 50 mM potassium phosphate (pH
Cloning, over-expression and purification of Nucleotide binding domain (Nbd) of EhPGDH: The Nucleotide binding domain (Nbd) (Arg^{103} - Gly^{285}) of EhPGDH encoded by a 489 base pair (bp) gene fragment was PCR amplified from the full length gene of EhPGDH (pET15b-EhPGDH). The PCR was performed with primers (forward-5' - GCTAGCATGAGGAATGGTGTCGAGAACTITGT-3' and reverse-5' - AAGCTITCCTATTITAATTGGTGTTGCAAAGAATCTTCC-3') with Nhel and HindIII restriction sites. PCR conditions used were: 1X94 °C for 5 minutes, 30X94 °C for 45 seconds, 51 °C for 45 seconds and 72 °C for 2 minutes and finally 1X 72 °C for 10 minutes. The amplified fragment was cloned in pTZ57R/T (InstAclone™ PCR cloning kit, Fermentas) cloning vector. The resultant construct was subsequently double digested with the respective restriction enzymes and the fragment (489 bp) obtained was further sub cloned into pET23a (+) vector (Novagen) between Nhel and HindIII sites. The resultant constructs were transformed into E coli Rosetta™ (DE3) pLysS competent cells for expression. A single colony of LB ampicillin plate was inoculated into 5 mL of LB broth (HI media) having ampicillin at a concentration of 100 μg/mL and allowed to grow overnight at 37 °C. It was then sub cultured in a 500 mL LB broth at 37 °C until A_{600} of 0.6 was achieved. The culture was then induced with 1mM of isopropyl-1-thio-β-D-galactopyranoside and was further incubated at 20 °C for 7 hours. The cells were harvested at 8000 rpm for 10 minutes and the resultant pellet was resuspended in 50 mM potassium phosphate (pH 8) buffer containing 300 mM NaCl, 2 mM PMSF, 5 mM imidazole, 1 mM DTT and 10 % glycerol. Cells were disrupted using a probe type sonicator and centrifuged at 13500 rpm for 30 minutes. The supernatant was applied on nickel nitrilotriacetic acid (NiNTA) agarose affinity column pre equilibrated with 50 mM potassium phosphate (pH 8) buffer along with 300 mM NaCl and 5 mM imidazole. The column was subsequently washed with two column volumes of the same buffer containing 5 mM and one column volume each of 20 mM and 40 mM imidazole, respectively. The protein was eluted using 500 mM imidazole. The eluted protein was tested for purity by SDS-PAGE and ESI-MS and was found to be >95 % pure.
**GdnHCl denaturation:** 3 µM protein (EhPGDH and Nbd) was denatured by method previously described in chapter 4 in potassium phosphate buffer (50 mM, pH 7.5) in presence of increasing concentration of GdnHCl.

**Spectroscopy:** Fluorescence spectra were recorded on Perkin Elmer LS50b luminescence spectrometer at 25 °C. For aromatic amino acid fluorescence an excitation wavelength of 280 nm and emission 300 nm and 400 nm were used. Tryptophan fluorescence anisotropy for the reduced cofactor was measured at a fixed wavelength of 295 nm and excitation wavelength was fixed at 335 nm. CD experiments were performed by method previously described in chapter 3. The thermal denaturation was carried out by monitoring changes in the CD ellipticity at 222 nm at constant rate of 1 °C /minute. Coupled enzyme reactions of protein-protein complex was measured as described earlier for the single EhPGDH protein in chapter 6 on Shimadzu UV 1650PC spectrophotometer at 25 °C.

**Pull-down assay:** Purified GST-EhPGDH and EhPSAT-His6 were incubated for 30 minutes in 0.5 mL reaction mixture at 4 °C, for complex formation. Mice anti-GST antibody (Sigma) was then added into the milieu and the reaction was further incubated for 1 hour. After addition of 5 mg of protein A-Sepharose beads (Sigma), the proteins were incubated for an additional 15 minutes. The immune-complex was precipitated and washed with ice-cold reaction buffer three times, suspended in loading buffer, and resolved on 12% SDS-PAGE.

**Size exclusion chromatography (SEC):** Equimolar samples of EhPGDH and EhPSAT were incubated for 30 minutes for complex formation. Subsequently, gel filtration experiments were carried out on a Superdex™ 200, 10/300GL column on AKTA FPLC (GE Health care). SEC for the Nbd was carried out on a Superdex™ 75, 10/300GL column on AKTA FPLC (GE Health care). The columns were pre-equilibrated and run with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 0.05 mL/minute, with detection at 280 nm.

**Glutaraldehyde Cross-linking:** The cross-linking of native EhPGDH, Nbd and the EhPGDH-EhPSAT complex was carried out as described earlier in chapter 3 and analyzed on 12 % SDS-PAGE. For the protein-protein complex the cross-linked samples were analyzed on 8 % SDS-PAGE.

**Molecular docking:** The homology models of EhPSAT and EhPGDH generated using Modeller program (Sali and Blundell 1993). They were further used as starting structures for computational docking experiments. The model of the bound protein complex structure from its unbound components was generated in a two-step docking procedure. Initially, Patchdock (Schneidman-
Duhoynv et al. (2005) was used to perform a global search to specifically predict protein-protein interaction site. Protein-protein interaction information was then used to predict EhPSAT-EhPGDH complex using RosettaDock (Gray et al. 2003a). In the first stage of the algorithm, RosettaDock employs a rigid-body Monte Carlo search, translating and rotating the one docking partner around the surface of the other, using residue-scale interaction potentials (Gray et al. 2003a; Simons et al. 1997; Simons et al. 1999). After this low-resolution search, explicit side chains are added to the protein backbones using a backbone-dependent rotamer packing algorithm (Gray et al. 2003b). A Monte Carlo-plus-minimization scheme then efficiently samples a set of local minima in a small region of docking conformation space by simultaneously optimizing the side-chain conformations and the rigid-body position. The search procedure is repeated from different random starting orientations to create \( \alpha(10) \) structures, which are then ranked using an energy function dominated by van der Waals interactions, an implicit solvation model (Lazaridis and Karplus 1999) and an orientation-dependent hydrogen bonding potential (Kortemme et al. 2003). In total, 1000 protein-protein complex decoys were generated at the end of the high-resolution search. These 1000 protein-protein decoys were then clustered on the basis of pair-wise rmsd using a Rosetta's clustering algorithm. The lowest energy structure in the cluster with the most population was selected as the final docking complex to run the MD simulations.

**Molecular dynamics simulation:** A 10 ns Molecular dynamics (MD) simulations was carried out for the lowest energy structure of most populous cluster produced by RosettaDock. The method used was same as previously described in chapter 5.

### 7.3 Results and Discussion

**EhPGDH production and oligomeric state**

EhPGDH was overproduced and purified by method described earlier in chapter 6. The purified protein was homogenous as indicated by a single protein band on SDS-PAGE and a single peak in ESI-MS of molecular mass about 35 kDa (data not shown). The molecular mass of EhPGDH under non dissociating conditions (using SEC) was approximately 70 kDa (elution volume 14.3 mL), suggesting stabilization of the protein as a dimer (Fig. 7.1C). This was further confirmed by glutaraldehyde mediated chemical cross-linking, where a single protein band corresponding to a molecular mass of about 70 kDa was observed on 12 % SDS-PAGE (Fig. 7.1D).
**Structural Features of EhPGDH**

**Secondary structure:** EhPGDH protein showed a typical α/β type secondary structure by far-UV CD spectrum (Fig.7.1A). The nucleotide binding domain (Nbd) of EhPGDH consists of residues Asn99 to Gly282 and contains six parallel β-strands interconnected with seven α-helices. This is a typical Rossman fold arrangement characteristic of dehydrogenases. The nucleotide-binding domain and substrate-binding domain form the active site cleft. The presence of the active site in this cleft is further supported by the presence of the nucleotide-binding site fingerprint (Gly-Xaa-Gly-Xaa2-Gly-Xaa17-Asp) located between amino acids 139–162 of EhPGDH (Ali et al. 2004a).

**Fluorescence resonance energy transfer:** The cofactor NADH is attached to the nucleotide binding domain of EhPGDH which contain five tyrosine residues. Fig.7.1B summarizes the result of FRET.
experiment on EhPGDH. For the fluorescence scan of native protein two emission maxima at 336 nm (corresponding for the aromatic amino acids) and 470 nm (corresponding to NADH) resulting from resonance energy transfer were observed. However, unfolding of the protein on incubation in 6 M GdnHCl resulted in a single emission maximum at 351 nm (data not shown). These observations demonstrate that in the native conformation of the protein the two fluorophores (NADH and tyrosine) are in close proximity (max. of about 5 Å) in the protein, and on denaturation these two fluorescent moieties move apart resulting in the loss of FRET.

**EhPGDH physically interacts with EhPSAT under in vitro conditions**

**Pull-down assay:** To analyze presence of physical interaction between GST-EhPGDH and EhPSAT-His₆, we incubated the interacting partners under *in vitro* conditions. Mouse anti-GST antibody was used for the pull-down experiment. The antibody was able to pull both the protein when added to the interacting mixture. However, it was unable to precipitate the non-cognate protein in the absence of the cognate partner. In a similar experiment with purified GST protein and EhPSAT, only GST was pulled out from the reaction mixture and EhPSAT was left in the milieu (Fig. 7.2). These results indicate specific protein-protein interactions between EhPGDH and EhPSAT. To further substantiate this indication we carried out gel filtration and chemical crosslinking experiment.

![Fig. 7.2: SDS-PAGE showing binding of EhPSAT-His6 specifically with GST-EhPGDH assessed by the pull-down assay using anti-GST antibodies. For these experiments, equimolar amounts of EhPSAT-His6 and GST-EhPGDH or GST were incubated in 50 mM phosphate buffer (pH 7.5) for 30 min. It was mixed with anti-GST antibodies for 1 h and then with Protein A-Sepharose beads for 15 min. The mixture was centrifuged and the beads were washed two times and proteins absorbed on beads were eluted by SDS-PAGE sample buffer and subjected to analysis. Lanes 1–4 represent mol. wt markers, and the purified proteins used during the pull down experiment; Lane 2, EhPSAT-His6 (43 kDa); Lane 3, GST-EhPGDH (66 kDa) and Glutathione-S-transferase GST (~26 kDa). Lanes 5–14 are the proteins after pull-down using...](image-url)
anti-GST antibodies. P represents proteins on protein A-sepharose beads and represents unbound proteins in the supernatant fraction.

**Gel filtration:** To analyze the stability of the EhPGDH-His$_6$EhPSAT-His$_6$ protein complex we carried out SEC of the complex on a Superdex™ 200 10/300 GL column (manufacturer protein exclusion limit of 600 kDa). The column was equilibrated and run in 50 mM potassium phosphate at pH 7.5 and was pre-calibrated using Ferritin (440 kDa), Aldolase (158 kDa), Conalbumin (75kDa) and Albumin (43 kDa) as molecular weight markers and Blue dextran as a marker of the excluded volume. A 0.6 mL sample containing equimolar amount of the interacting proteins was incubated for complex formation prior to being applied on the column. The enzymes co-eluted at an estimated molecular weight of about 155 kDa (Fig. 7.3A). Fractions eluted were assayed for the presence of aminotransferase and dehydrogenase activities.

**Chemical cross-linking:** The eluted protein fraction from the SEC experiment was subjected to glutaraldehyde mediated chemical cross-linking. The cross-linked protein-protein complex was resolved on an 8% SDS-PAGE and a protein band of approximately 155 kDa was obtained corresponding for the EhPGDH-EhPSAT complex (Fig.7.3B). The above results suggest that EhPSAT and EhPGDH form a stable complex.

![Fig. 7.3: SEC and cross-linking profile of the PGDH and PSAT complex.](image)

(A) SEC profile of EhPGDH, EhPSAT and protein-protein complex on a Superdex™ 200 10/300 GL column.
(B) A curve of elution volume plotted against standard molecular mass markers. The proteins are (1) 440KDa (ferritin), (2) 158KDa (aldolase), (3) 75KDa (conalbumin) and (4) 43KDa (ovalbumin). The complex eluted at ~155 kDa.
(C) 8% SDS-PAGE profile of glutaraldehyde cross-linked sample of PGDH-PSAT complex. Lanes 1-3 uncross-linked EhPSAT, EhPGDH and glutaraldehyde cross-linked protein-protein complex, respectively.
Tryptophan fluorescence anisotropy: To analyze the binding constant of the PSAT-PGDH complex we carried out fluorescence studies. Fluorescence anisotropic studies are ideally suited for studying interacting systems where one of the components is tagged with a fluorescent probe (Hearl and Churchich 1984). Tryptophan is an intrinsic fluorophore which has been frequently used for monitoring changes in the tertiary micro environment of the proteins as a consequence of physical interactions. Interestingly, EhPGDH has no tryptophan moiety however; EhPSAT has three tryptophan residues present in the large PLP-binding domain close to the active site. Fig. 7.4A shows the increase in fluorescence anisotropy when a sample of EhPSAT-His<sub>6</sub> at a concentration of 1μM was allowed to interact with increasing concentrations of EhPGDH-His<sub>6</sub> at pH 7.5 in 50 mM phosphate buffer.

The plot of anisotropy versus concentration of EhPGDH, was used to infer apparent dissociation constant of the complex. The K<sub>d</sub> value of 3.453 x 10<sup>-7</sup> M was observed as a result of binding of PSAT to PGDH.

![Anisotropy and computational docking and energy minimized structure of protein complex.](image)

Fig. 7.4: Anisotropy and computational docking and energy minimized structure of protein complex.

(A) Titration of EhPSAT with increasing concentration of EhPGDH. Fluorescence anisotropy signals are represented in absence ( ) and presence (o) of 200 mM NaCl.

(B) Molecular docked and energy minimized model of EhPGDH and EhPSAT complex. The EhPGDH dimer is represented by magenta and cyan ribbons while the EhPSAT dimer is represented by green and red ribbons, respectively. Inset shows a magnified view of the protein-protein complex interface.

Complex docking and Molecular dynamics simulation: In order to understand the interaction which stabilize the EhPSAT-EhPGDH complex, we generated a model for the complex between EhPSAT...
and EhPGDH using the rigid-body Monte Carlo search employed by protein-protein docking program, RosettaDock which combines Monte Carlo-plus-minimization scheme with van der Waals interactions, implicit solvation, and hydrogen bonding potential dominated energy function. The 10 top ranked members with lowest energy are shown in Fig. 7.5A.

The relatively small deviation in structures of most populous cluster and a well-defined docking funnel obtained after plotting the Rosetta total score and RMSD with lowest ranked structure indicates that the EhPSAT-EhPGDH predicted complex is well defined (Fig. 7.5B). In the lowest energy structure, 99.5% of the residues were in most favoured and allowed region on Ramachandran plot using program Procheck (Laskowski et al. 1996), 0.3% in the generously allowed region and 0.2% in the disallowed region, suggesting good quality of the predicted complex model. As Cluster 1 contains the largest number of members (46), the lowest energy structure from this cluster with Rosetta all atom total energy of -2387.98 was selected to represent model of EhPSAT-EhPGDH complex.

Fig. 7.5: Complex docking.
(A) Top ranked conformation ensemble of EhPSAT-EhPGDH complex. The EhPGDH dimer is represented by magenta and cyan ribbons while the EhPSAT dimer is represented by green and red ribbons, respectively.
(B) Rosetta total scores for generated protein-protein decoys.

The energetic and structural properties were monitored along the entire MD trajectory of the complex. The average energy of the entire system as a function of simulation time for EhPSAT-EhPGDH complex was found to be -369619.6862 Kcal mol⁻¹ and the average fluctuation from the average total
complex was found to be -369619.6862 Kcal mol\(^{-1}\) and the average fluctuation from the average total energy was less than 2000 Kcal mol\(^{-1}\) (data not shown). Thus the total energy is oscillating around the average energy, showing the stability of system at a given temperature of 300 K.

Stability of the complex structure can be also reflected by its deviation from the initial structure in terms of root mean square deviation (RMSD) of, e.g., the backbone atoms of a protein over the course of a simulation and may be used as a measure of the conformational stability of a protein structure or model during that simulation. Therefore, we monitored the RMSD of backbone atoms throughout the trajectory from their initial configuration. The initially RMSD from starting structure rose from 1 to 5 Å till 8 ns. This initial rise is commonly observed in protein simulations of this much size and is thought to reflect the relaxation of the protein once constraints applied at the time of heating and equilibrations are removed. After 8 ns the relative fluctuation for the RMSD is small (less than 1 Ångstrom) as system has been stabilized (Fig. 7.6). From the structural and energetic analysis, we can see that the system is stable from both the rmsd and binding energies from the 10 ns molecular dynamics simulation.

![Graph showing RMSD over time](image.png)

**Fig. 7.6:** Root mean square deviation (rmsd) of the backbone atoms of the EhPSAT-EhPGDH complex with respect to the first snapshot as a function of time.
MM-PBSA and MM-GBSA approach was then used to calculate the free energy change due to the formation of EhPSAT-EhPGDH complex. The snapshot structures used for MM-GBSA and MM-PBSA calculation were extracted from molecular dynamics simulation trajectory and last 2 ns used for the calculation of binding free energy. The major contributions to the binding free energy arise from the polar contribution to solvation energy or electrostatic solvation $\Delta E_{\text{polar}}$ and $\Delta E_{\text{polar}}$, as calculated by MM-GBSA and MM-PBSA approach (-67.6851 and -89.6863 kcal/mol). The highly favorable solvation energy probably reflects the great number of favorable interactions from the positively and negatively charged residues at the binding interface. Apart from this, the intermolecular van der Waals interactions with a value of -50.1361 kcal mol$^{-1}$ also provide the driving force for binding and formation of the protein-protein complex. The nonpolar contribution to solvation energy were also found to make favorable contribution to the binding energy with of $\Delta E_{\text{nonpolar}}$ of -6.5842 kcal mol$^{-1}$ and $\Delta E_{\text{nonpolar}}$ of -5.9895 kcal mol$^{-1}$ based on the MM-GBSA and MM-PBSA methods, respectively.

![Free energy decomposition profile of PSAT-PGDH complex](image)

Fig. 7.7: Free energy decomposition profile of PSAT-PGDH complex.
Furthermore, in order to gain a clear picture about the contribution of each residue at the interface that make significant contributions to the binding free energy associated with complex formation, the total binding free energy was decomposed into per residue contributions, using MM-GBSA free energy decomposition analysis. The residue wise contribution and the amino acid residues that are most important contributors for the interaction between EhPSAT and EhPGDH are shown in Fig. 7.7. In general, Glu131, Gln133, Pro135, Asp137, Tyr138, Asp139, Glu159, Ser160, Pro161 of PSAT and Phe122, Lys123, Phe249, Phe253 and Arg256 of PGDH showed most significant and favorable contributions for complex formation (Fig. 7.4 B inset).

**EhPGDH has two independent folding/unfolding domains**

**Thermal denaturation:** The thermal unfolding of EhPGDH was characterized by monitoring the loss of secondary structure of enzyme with temperature. Fig. 7.8A summarizes the changes in CD ellipticity at 222 nm of EhPGDH with increasing temperature. Two distinct transitions between 25 and 100 °C were observed for the protein. These transitions were in the temperature regions from 35 to 60 °C and 65 to 100 °C and centered at about 45 and 65 °C, respectively.

**GdnHCl unfolding:** The guanidine hydrochloride-induced changes in the structural properties of EhPGDH were studied using CD spectroscopy. The effect of GdnHCl on the secondary structure of EhPGDH was studied by monitoring changes in the far-UV CD profile of the enzyme. Fig. 7.8B summarizes the effect of increasing GdnHCl concentration on loss of ellipticity at 222 nm for EhPGDH. Two well separated transitions, a sharp transition between 0.4 and 0.8 M GdnHCl, and a broad transition between 0.9 and 3 M GdnHCl were observed. Furthermore, only about 30% loss of secondary structure was found to be associated with the first transition. The unfolding curve of EhPGDH with two transitions indicates presence of two independent structural domains.

![Fig. 7.8: Denaturation profile of EhPGDH. (A) Thermal denaturation profile of EhPGDH (●) and Nbd (○). (B) GdnHCl-induced denaturation profile of EhPGDH (●) and Nbd (○).](image)
The nucleotide binding domain (Nbd) of EhPGDH: The results from the thermal and chemical unfolding studies of EhPGDH show that the two domains the nucleotide binding domain (Nbd) and the substrate binding domain (Sbd) probably unfold independent of each other. This shows that possibly the two domain fold/unfold independently. Since MD simulations analysis clear suggest that EhPGDH interacts exclusively through its nucleotide binding domain (Nbd) with EhPSAT. The Nbd of EhPGDH was over-expressed and purified for further analysis. The purified protein has ~18.5 kDa of molecular mass as observed from the SDS-PAGE analysis (Fig.7.9A) and ESI MS (data not shown). Under non-dissociating conditions the domain exist as a dimer as observed by SEC (Fig. 7.9D). Similar results were also obtained by glutaraldehyde mediated protein crosslinking where a single band of approximately 38 kDa was obtained on a 12% SDS-PAGE (Fig. 7.9D inset).

Structural characterization of Nbd: The recombinant domain has a typical α/β type secondary structure (Fig 7.9 B). The fluorescence emission spectrum of EhPSAT-Nbd showed an emission maximum of about 338 nm (Fig. 7.9 C). The purified Nbd was functionally inactive as the active site pocket of the protein is situated at the interface of the Nbd and the substrate binding domain (Sbd). The thermal unfolding of Nbd as monitored by the loss of secondary structure of the domain with temperature show a single transition with a Tm of approximately 65 °C (Fig. 7.8 A). This result was quite comparable to the second transition of the thermal denaturation curve of the native EhPGDH. These observations suggest that the higher transition observed during the thermal unfolding of EhPGDH corresponds to melting of Nbd of the enzyme.

GdnHCl-induced unfolding of the Nbd also showed a single transition, as monitored by the loss of CD ellipticity at 222 nm (Fig. 7.8 B). This transition overlapped the second phase transition of the GdnHCl-induced unfolding curve of the native EhPGDH. The Cm value for the domain was about 1.8 M, which was quite comparable to the Cm observed for the second transition of the biphasic curve during urea denaturation of the EhPGDH. These results show that Nbd maintain its conformation even when it's been isolated and is an independent folding/unfolding domain of EhPGDH.

The isolated Nbd interacts with EhPSAT: Complex docking analysis showed that the Nbd of EhPGDH interacts with the EhPSAT. Subsequently binding study was performed between the Nbd and EhPSAT using fluorescence anisotropy. Incubation of increasing concentration of Nbd with EhPSAT showed an exponential enhancement in the anisotropy values. Based on the results the dissociation constant calculated was quite similar to that observed for the native EhPGDH-EhPSAT protein complex. The experiments clearly suggest that the Nbd specifically interacts with the
EhPSAT. Thus, the nucleotide binding domain of the EhPGDH specifically interacts with the EhPSAT to form a protein-protein complex.

![SDS-PAGE profile of over-expressed and purified Nbd](image1)

**Fig. 7.9:** Over-expression, purification and structural characterization of Nucleotide binding domain (Nbd) of EhPGDH.

(A) 12 % SDS-PAGE profile of over-expressed and purified Nbd. Lanes 1-3 represent mol wt markers, *E coli* BL-21 cell lysate with over-expressed protein and purified Nbd, respectively.

(B) Far-UV CD profile of Nbd.

(C) A fluorescence emission spectrum of Nbd. Excitation was at 280 nm and emission recorded from 300 nm to 400 nm.

(D) SEC profile of Nbd with absorbance at 280 nm (solid line) on a superdex™ 75 10/300 GL column. Inset shows a 12 % SDS-PAGE profile of glutaraldehyde cross-linking experiment. Lanes 1-3 represent mol. wt markes, uncross-linked Nbd and cross-linked protein, respectively

(E) Titration of EhPSAT with increasing concentration of EhPGDH-Nbd. Fluorescence anisotropy signals are represented in (o).

**Ionic interactions are involved in EhPGDH-EhPSAT for complex formation:** The EhPSAT-EhPGDH complex shows presence of ionic residues at the complex interface which might play a major role in complex formation (Fig. 7.4 B inset). To further validate this we performed pull down assay of EhPSAT and EhPGDH in presence of increasing concentration of NaCl. The two interacting partners stayed with each other at lower NaCl concentrations however; at 200 mM NaCl the interaction was lost as observed by the presence of the two interacting proteins separated in pellet and supernatant fractions on SDS-PAGE (data not shown). Similar results were observed with
fluorescence anisotropy (Fig. 7.4 A), SEC and chemical cross-linking (data not shown) in presence of 200 mM NaCl. Thus ionic interactions are playing a major role in complex formation and on increasing NaCl concentration the interactions are compromised resulting in loss of complexation.

**Substrate channelling:** Enzymes efficiently work in close association with each other. This micro compartmentation leads to substrate channelling in which the intermediate produced by one enzyme is transferred to the next enzyme without being completely mixed with the bulk phase (Datta et al. 1985; Spivey and Ovadi 1999). Such substrate channelling is observed for enzymes involved in metabolic pathways and the phenomenon ensures optimal metabolon activity without diffusion of the pathway intermediates in milieu. The process occurs because of close proximity (40 to 60 Å) of the active sites of the interacting enzymes (Spivey and Ovadi 1999). The structure of the EhPGDH-EhPSAT enzyme complex shows that active sites of the two enzymes are closely associated. The EhPGDH-EhPSAT complex showed as Km of 100 μM for 3-PGA which was significantly lower than that observed for the individual PGDH enzyme (225 μM). The results suggest that the product of PGDH catalyzed reaction is been taken up by its interacting partner PSAT which in turn shifts the reaction equilibrium in forward direction. Phosphoserine, the final product of the enzyme complex can be taken up by phosphoserine phosphatase (PSP) or cysteine synthase (CS) in their respective committed reactions for the formation of L-serine or L-cysteine().
7.4 Summary

D-phosphoglycerate dehydrogenase (PGDH) and phosphoserine aminotransferase (PSAT) are the first two enzymes of the phosphorylated serine biosynthesis pathway. For *Entamoeba histolytica* this pathway is situated upstream and is a very crucial part of vital cysteine metabolism. The present study provides an evidence of existence of physical interactions between *E. histolytica* PGDH and PSAT. Pull-down assay shows that the EhPGDH specifically interacts with EhPSAT. The fluorescence anisotropy experiments show that the two proteins interact in 1:1 stoichiometry. A complex of ~155 kDa was stabilized at neutral pH as shown by SEC and chemical cross-linking experiments. Each subunit of EhPGDH is composed of two structural domains the nucleotide binding domain (Nbd) and the substrate binding domain (Sbd). Molecular docking and MD simulations were used to develop an energy minimized model of the complex which showed that the Nbd of EhPGDH specifically interacts with the EhPSAT. The study also showed that ionic interactions play a predominant role in complex formation. Denaturation experiments of EhPGDH suggest that the two domains can fold/unfold independently. Furthermore the Nbd was cloned over-expressed and purified to homogeneity. The purified domain existed as a dimer and specifically interacted with EhPSAT as shown by fluorescence anisotropy. The EhPGDH-PSAT complex showed presence of efficient substrate channelling between the two enzymes.
Entamoeba histolytica Phosphoserine aminotransferase (EhPSAT): insights into the structure-function relationship

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Abstract

Background: Presence of phosphorylated Serine biosynthesis pathway upstream to the de novo cysteine biosynthesis pathway makes PSAT a crucial enzyme. Besides this, phosphoserine produced by the enzyme can also be taken up directly by cysteine synthase as a substrate. PSAT is a PLP dependent enzyme where the cofactor serves as an epicenter for functional catalysis with the active site architecture playing crucial role in optimum function of the enzyme.

Findings: EhPSAT is a homodimer of molecular mass 86 kDa. To understand the structural modulations associated with pH dependent changes in functional activity of EhPSAT detailed biophysical studies were carried out. pH alterations had no significant effect on the secondary structure, cofactor orientation and oligomeric configuration of the enzyme however, pH dependent compaction in molecular dimensions was observed. Most interestingly, a direct correlation between pH induced modulation of functional activity and orientation of Trp 101 present in the active site of the enzyme was observed. Sodium halides nullified the pH induced global changes in the enzyme, however differential effect of these salts on the active site microenvironment and functional activity of the enzyme was observed.

Conclusions: The study unequivocally demonstrates that pH induced selective modification of active site microenvironment and not global change in structure or oligomeric status of the enzyme is responsible for the pH dependent change in enzymatic activity of PSAT.

Background

PSAT is a vitamin B6-dependent enzyme that belongs to the α-family of pyridoxal-5′-phosphate (PLP) enzymes. It catalyzes the reversible conversion of 3-phosphohydratepyruvate to L-phosphoserine in a glutamate linked transamination reaction, the second step of phosphorylated serine bio-synthetic pathway. Earlier studies on PSAT from E. coli [1], Bacillus alcalophilus [2], Arabidopsis thaliana [3], and Homo sapiens [4] suggests that the enzyme exist as a homodimer with a subunit molecular mass between 40 to 48 kDa. Each subunit is predominantly composed of two domains, a large PLP binding domain and a small domain comprising of C-terminal part along with a short N-terminal portion [1].

Structurally PSAT is a α/β protein with one PLP molecule present in the active site, per monomer. The dimeric configuration of the enzyme is essential for the functional activity [5]. The active site amino acid residues are nearly conserved in all PSATs (Fig. 1A).

Entamoeba histolytica is a protozoan parasite that infects the gastrointestinal tract and causes amoebic colitis and extra intestinal abscesses in humans [6]. Role of L-serine in a number of important metabolic pathways in the parasite has been well established [7,8]. Predominantly it serves as a precursor molecule for L-cysteine biosynthesis which plays important role in survival, growth, attachment [9,10], anti-oxidative defense [11], and Fe-S cluster biosynthesis [12]. From the amino acid sequence alignment and phylogenetic analyses EhPSAT shows close association with bacterio-ide PSAT [13].
Biophysical characterization of *Entamoeba histolytica* phosphoserine aminotransferase (EhPSAT): role of cofactor and domains in stability and subunit assembly

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Abstract We investigated the role of the cofactor PLP and its binding domain in stability and subunit assembly of phosphoserine aminotransferase (EhPSAT) from an enteric human parasite *Entamoeba histolytica*. Presence of cofactor influences the tertiary structure of EhPSAT because of the significant differences in the tryptophan microenvironment and proteolytic pattern of holo- and apo-enzyme. However, the cofactor does not influence the secondary structure of the enzyme. Stability of the protein is significantly affected by the cofactor as holo-enzyme shows higher $T_m$ and $C_m$ values for thermal and GdnHCl-induced denaturation, respectively, when compared to the apo-enzyme. The cofactor also influences the unfolding pathway of the enzyme. Although urea-dependent unfolding of both holo- and apo-EhPSAT is a three-state process, the intermediates stabilized during unfolding are significantly different. For holo-EhPSAT a dimeric holo-intermediate was stabilized, whereas for apo-EhPSAT, a monomeric intermediate was stabilized. This is the first report on stabilization of a holo-dimeric intermediate for any aminotransferase. The isolated PLP-binding domain is stabilized as a monomer, thus suggesting that either the N-terminal tail or the C-terminal domain of EhPSAT is required for stabilization of dimeric configuration of the wild-type enzyme. To the best of our knowledge, this is a first report investigating the role of PLP and various protein domains in structural and functional organization of a member of subgroup IV of the aminotransferases.

Keywords EhPSAT • *Entamoeba histolytica* • Phosphoserine aminotransferase • Pyridoxal-5'-phosphate • Denaturation • PLP-binding domain

Abbreviations

- PSAT: Phosphoserine aminotransferase
- PLP: Pyridoxal-5'-phosphate
- Ni-NTA: Nickel nitrilotriacetic acid
- SEC: Size exclusion chromatography

Introduction

More than 30% of the known proteins require cofactors for their ideal biological function (Deu and Kirsch 2007a). In some cases the cofactor guides the folding pathway of protein by binding to the partially unfolded state or associating with the native conformation of the protein (Bollen et al. 2005; Muralidhara and Wittung-Stafshede 2005). For large multi-domain and oligomeric proteins, the cofactor stabilizes its binding domain (Wardell et al. 2005) or the quaternary structure if present at the subunit interface (Pant and Crane 2005). However, for certain proteins the cofactor interacts with the