Entamoeba histolytica D-Phosphoglycerate dehydrogenase:

Role of Glu 108 in subunit assembly and dimer stability
6.1 Introduction

D-phosphoglycerate dehydrogenase (PGDH) belongs to 2-hydroxy acid dehydrogenase family of proteins. It is ubiquitously present in all organisms in which the phosphorylated serine biosynthesis pathway serves as a major source of L-serine flux in the cell. The protein shows considerable degree of functional (inhibition by L-serine) and structural variations from organism-to-organism (Dey et al. 2005b). The concentration of L-serine that produces half-maximal inhibition of catalytic activity for PGDH from *E. coli*, *M. tuberculosis*, *C. glutamicum*, and *B. subtilis* are ~3, 30, 640, and 10,000 μM, respectively (Dey et al. 2005a; Dey et al. 2005b). However, *Entamoeba histolytica* PGDH (EhPGDH) is insensitive to L-serine induced inhibition of the catalytic reaction (Ali et al. 2004a).

PGDH has three structural domains: a substrate binding domain, a nucleotide binding domain and a C-terminal regulatory domain. PGDH of some bacteria and certain lower eukaryotes, such as yeast, *Leishmania*, and *Neurospora*, are structurally similar to the *E. coli* enzyme where the regulatory domain is involved in effector (L-serine) binding and regulation of activity (Dey et al. 2005b). However, at certain instances such as *Mycobacterium*, *Bacillus subtilis*, *Corynebacterium*, plants such as *Arabidopsis*, and higher order eukaryotes, including mammals an additional intervening domain is present sandwiched between the substrate-binding domain and the regulatory domain (Dey et al. 2005b). PGDH generally exist as a tetramer *i.e* dimer-of-dimer however, there are some exceptions. The nucleotide binding domain forms the interface where two adjacent subunits interact to form a protein dimer. The regulatory domain and a conserved tryptophan residue of the nucleotide binding domain of two such dimers interact to stabilize the tetrameric quaternary structure configuration of PGDH (Grant et al. 2000).

Recent studies show that the enteric human parasite *Entamoeba histolytica* possess a highly unusual PGDH with diverse structural and functional features as compared to its counterparts from other organisms (Ali et al. 2004a). The enzyme exists as homodimer where each subunit of the protein has only two domains, a nucleotide binding domain and the substrate binding domain (Fig. 6.1). EhPGDH lacks the regulatory domain, which is implicated for allosteric inhibition by L-serine and tetramerization. EhPGDH also lacks the important tryptophan residue in its nucleotide binding domain which plays a crucial role in stabilization of a tetrameric quaternary structure (Ali et al. 2004a). Although much has been deciphered about the structural features of PGDH in terms of the role played by different domains in the functional activity and allostery associated with L-serine induced inhibition, no information is available about its monomer-monomer interactions especially the forces that stabilize the subunit assembly.
The dimeric structural organization of EhPGDH makes it an ideal candidate for studying subunit interactions at the dimer interface, which to the best of our knowledge is never been attempted previously. In the present study non-covalent interactions which stabilize the native (dimeric) conformation of the protein are identified. Mild acidic conditions were employed as a tool to perturb the electrostatic interactions and to gather information about the microenvironment at the subunit interface. The studies show that EhPGDH dimer dissociates at pH 5 with significant alterations in the functional and structural features of the protein. Homology modeling and molecular dynamic simulations are used to identify the amino acid residues at the subunit interface which might be involved in dimer stability. Key residues were carefully selected and site-directed mutagenesis study was performed to establish their possible role in stabilization of the protein dimer. Glu108 forms a salt bridge with Arg119 of the neighbouring subunit, at the dimer interface. The E108A mutant of EhPGDH was stabilized as a monomer at pH 7. Thus, the Glu108 is important for dimer stability.
opportunity to understand the mechanism that works at the subunit interface which stabilizes the protein dimer.

6.2 Materials and Methods

Materials

All chemicals used in the study were purchased from Sigma-Aldrich chemical company St Louis, USA, and were of highest purity available. Size exclusion chromatographic (SEC) column was purchased from GE Healthcare Bioscience and Ni-NTA agarose was from Quiagen. Milli Q water was used for all experimental work.

Methods

Over-expression and Purification of EhPGDH: The over-expression and purification of recombinant EhPGDH was carried out as described earlier (Ali et al. 2004a).

Cloning, over-expression and purification of E108A EhPGDH mutant: The E108A mutant of EhPGDH was generated using the GeneTailor™ site directed mutagenesis system (Invitrogen) from EhPGDH gene harboured in a pET23a (+) vector (Novagen) between Nhel and Hindlll restriction sites. PCR was performed with primers (forward-S' CAAAATAGGAATGGTGTTGCAGCACTTTGGATGATG-3' and reverse-S' CATCATCCCCAATACAAAGTGCTGCAACACCACATTCCATTTTG-3'). PCR conditions used were: 1X 94 °C for 5 minutes, 30X 94 °C for 45 seconds, 54.5 °C for 45 seconds and 68 °C for 3 minutes and finally 1X 72 °C for 10 minutes. The resultant construct was sequenced to confirm the point mutation at 108 amino acid position of EhPGDH.

The E108A-EhPGDH plasmid was transformed into E coli BL-21 competent cells for expression. A single bacterial colony was inoculated into 5 mL of LB broth (HI media) having ampicillin at a concentration of 100 µg/mL and was 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 0.5 mM of isopropyl-1-thio-β-D-galactopyranoside and was further incubated at 16 for 5 hours. Cells were harvested at 8000 rpm for 10 minutes and the resultant pellet was suspended in 50 mM potassium phosphate (pH 8) buffer containing 300 mM NaCl, 2 mM PMSF and 10 % glycerol and were disrupted using a probe type sonicator and centrifuged at 13500 rpm for 30 minutes. The supernatant was applied on nickel nitrilotriacetic acid (Ni-NTA) agarose affinity column pre-equilibrated with 50 mM potassium phosphate (pH 8) buffer along with 300 mM NaCl. The column
was subsequently washed with one column volume of the same buffer with 20 and 40 mM imidazole. The protein was eluted using 500 mM imidazole in the same buffer. The eluted fraction was extensively dialyzed in 50 mM phosphate buffer pH 7.0. The eluted protein was tested for purity by SDS-PAGE and ESI-MS and was found to be about 95% pure.

*Size exclusion chromatography (SEC):* Gel filtration experiments were carried out on a Superdex™ 75, 10/300GL column (manufacture's exclusion limit 75 kDa) on AKTA FPLC (GE Healthcare). The methodology used was same as described in chapter 3.

*Spectropolarimetric measurements:* Circular dichroism (CD) measurements were made on JASCO J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate with 3 µM protein by method previously described in chapter 3.

*Fluorescence spectroscopy:* Fluorescence spectra were by method previously described in chapter 3. Excitation wavelength was 274 nm and the spectra were recorded between 290 nm to 350 nm. Fluorescence polarization for the cofactor was measured at a fixed wavelength of 460 nm and excitation wavelength was fixed at 340 nm.

*Acrylamide quenching:* Quenching studies of the intrinsic protein fluorescence was performed on a Perkin Elmer LS50B luminescence spectrometer in a quartz cell of 5 mm path length using a stock solution of 5 M acrylamide. To fixed amount (5 µM) of protein pre-equilibrated with desired pH, increasing amounts of acrylamide (0.1–1 M) was added and the samples were incubated for 1 hour at 25 °C before the measurements were made. The excitation wavelength used was 274 nm. The emitted light intensity was integrated over the period of 1 s and detected at 290–350 nm. The results were analysed by Stern–Volmer and modified Stern–Volmer plots using the following equations (Lakowicz 2006):

\[
\frac{F_0}{F} = 1 + K_{sv} [Q] \quad (eq. 1)
\]

\[
\frac{F_0}{(F_0 - F)} = \frac{1}{f_a K_c [Q]} + \frac{1}{f_a} \quad (eq. 2)
\]

where \(F_0\) and \(F\) are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher, respectively, \(K_{sv}\) is the Stern–Volmer constant, \(f_a\) the fraction of the fluorophores accessible to the quencher, \(K_c\) the collisional quenching constant, and \(Q\) is the concentration of the quencher.

*Enzyme Activity:* The forward enzymatic activity of EhPGDH activity was assayed at 25 °C for 5 minutes in 10 mM CGH buffer of desired pH by method previously described by Ali and Nozaki.
Chemical Cross-linking: Glutaraldehyde mediated chemical cross-linking was performed by method previously described in chapter 3. The samples were analyzed on 12% SDS-PAGE.

Homology modeling and Molecular dynamic simulation: The three-dimensional model of the EhPGDH was built based on four PGDH crystal structure templates Escherichia coli, Protein Data bank code 2PA3, Mycobacterium tuberculosis, PDB code 1YGY, Pyrococcus horikoshii, PDB code 1WWK and Homo sapiens, PDB code 2G76 using Modeller program (Sali and Blundell 1993). Dimer structure of EhPGDH was prepared by superimposing on their corresponding template dimers. Subsequently, a 10 ns Molecular dynamics (MD) simulations was carried out for EhPGDH dimer by method previously described in chapter 5. The accessible surface area (ASA) was calculated with the help of MSMS program (Sanner et al. 1996).

6.3 Results and Discussion

To understand the correlation between folding and assembly of EhPGDH, it was important to track the relations between local and global changes that affect the subunit conformation and assembly. The balance of electrostatic interactions in the native state of the protein is affected by changing the pH of the solution, which results in local/global perturbations in the protein. Time-dependent changes in secondary and tertiary structure of EhPGDH at pH 5 and 7 were monitored by far-UV CD and intrinsic fluorescence to standardize the incubation time required for achieving equilibrium under these conditions. Under all the conditions studied, the changes occurred within a maximum of 1 hour with no further alteration up to 12 hours (data not shown). Based on these observations an incubation time of 2 hours was kept constant for the protein to achieve equilibrium under all pH values before the measurements were made.

Changes in molecular properties of EhPSAT associated with pH change

Enzymatic activity and cofactor polarization: Enzyme activity is regarded as the most sensitive probe to study the changes in enzyme conformation during various treatments as it reflects subtle readjustments at the active site, allowing very small conformational variations of an enzyme structure to be detected and analysed. Fig. 6.2A summarizes the effect of acidic pH on the enzymatic activity of EhPGDH. On decrease in pH from 7 to 4.5, a steep decrease in enzymatic activity was observed. The enzyme was completely inactive at pH 5. These observations demonstrate that the functional activity of EhPGDH is sensitive to acidic pH. Studies at pH below 5.0 could not be performed as the cofactor NAD'/NADH is unstable below this pH range (Fawcett et al. 1961). The modulation of functional activity of EhPGDH can be associated with small fluctuations close to or in the active site.
of the enzyme. The cofactor NADH is important for functional activity of the dehydrogenases as it plays a crucial role in proton relay system associated with the enzyme catalysed reaction. EhPGDH, in its native conformation has two NADH molecules associated with the two active sites of the enzyme dimer (Fig. 6.1). Hence, it was important to analyse the pH-induced changes in the NADH microenvironment of EhPGDH by studying the dynamics of the protein bound cofactor.

NADH has been used as a natural marker to probe active site microenvironment of the dehydrogenases. Fig. 6.2A shows the changes in NADH fluorescence polarization as a function of pH. A steep decrease in the polarization values was observed with decrease in pH from 7 to 4.5. The cofactor got released from the protein at pH 5 resulting in its free movement and loss of polarization. These results indicate that flexibility of the protein bound cofactor was significantly modulated in acidic conditions. The cofactor polarization followed similar trend as the enzymatic activity which suggest that the cofactor conformation plays a key role in maintenance of the enzymatic activity.

**Secondary Structure:** To study the pH-induced changes in the secondary structure elements of the EhPGDH, far-UV CD studies were carried out. Fig. 6.2B shows the effect of pH on CD signal at 222 nm for EhPGDH. No significant change in CD signal of the enzyme at 222 nm was observed between pH 8 and 6. Further decreasing the pH below 6 resulted in a slight decrease in the CD signal suggesting subtle changes in the secondary structure of the protein under acidic conditions between pH 5.5 and 4.

![Fig. 6.2: Changes in the functional and structural features associated with pH change.](image)

(A) pH-dependent enzymatic activity profile of EhPGDH. The data is been represented in percentage relative activity with activity at pH 7 taken as 100 % (o). The pH dependent changes in the fluorescence polarization of EhPGDH are shown in (•).

(B) pH-induced changes in the secondary structure of the EhPGDH, monitored by the CD-signal at 222 nm.
**Intrinsic fluorescence**: The spectral features of intrinsic fluorophores of a protein are dependent on the dynamic and electronic properties of the fluorophore microenvironment; hence, steady state fluorescence has been extensively used to obtain information on the structural changes of the protein (Mishra et al. 2011.; Semisotnov et al. 1991). Since no tryptophan residue is present in EhPGDH (Ali et al. 2004a), the intrinsic protein fluorescence was mainly due to tyrosine and phenylalanine residues. Each subunit of the EhPGDH dimer possesses seven tyrosine and fourteen phenylalanine residues. Intrinsic fluorescence by virtue of these fluorophores was used to monitor changes in the tertiary structure of the protein. Fig. 6.3A shows pH dependent changes in the intrinsic fluorescence intensity of EhPGDH. An initial increase in the intensity was observed between pH 7 and 5. The fluorescence intensity tends to remain constant between pH 5 and 4. The observed increase in fluorescence intensity can be attributed to significant changes in the tertiary structure at low pH exposing the buried fluorophores to polar solvent (Fig. 6.3A inset). A red shift was also observed in the emission maxima between pH 7 and 5. Tyrosine and phenylalanine residues are generally present buried in the hydrophobic core of a protein molecule. The observed increase in fluorescence intensity and the red shift in emission maxima of the protein under acidic conditions might be due to slight exposure of these moieties from the core to the polar environment. These findings were further investigated by quenching studies by a neutral quencher acrylamide.

![Fig. 6.3: Changes in the tertiary structure of the protein with pH changes.](image)

(A) pH-induced changes in the tertiary structure of the protein as monitored by pH-induced increase in the fluorescence intensity of EhPGDH. The inset shows fluorescence spectra at pH 7 and 5.

(B) Acrylamide quenching: Stern-Volmer and modified Stern-Volmer plots (inset) for EhPGDH at pH 7 (■) and pH 5 (●).

**Arylamide Quenching studies**: The fluorescence properties of aromatic amino acid residues can be used to obtain topological information of proteins. Fluorescence quenching of the intrinsic (amino acid) fluorophores by neutral quencher (acylamide) can be utilized as an useful tool to obtain information about the solvent accessibility of these residues in proteins and the polarity of their...
microenvironment, as it can discriminate between 'buried' and 'exposed' side chains (Lakowicz 2006; Pawar and Deshpande 2000). Its ability to collisionally quench the excited aromatic rings depends only on their 'exposure' to the quencher. Fig. 6.3B shows the Stern–Volmer plot for the acrylamide quenching studies performed on EhPGDH at pH 7 and 5. The fractional accessibility of the fluorophore to the quencher was calculated by the help of the modified Stern–Volmer plot (Fig. 6.3B inset).

The values of Stern–Volmer constant (Ksv) and fractional accessibility of the fluorophores to quencher (fₒ) were calculated from the above plots and presented in Table 6.1. The aromatic amino acids in the protein at pH 5.0 are more exposed to the polar solvent and thus are more accessible to the quencher as compared to the protein at pH 7.0. The results suggest that the tertiary structure of the protein is significantly perturbed under acidic conditions at pH 5. This can be attributed to changes in the balance of electrostatic interactions in the native structure of the protein as a result of protonation of charged amino acid residues at low pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>Ksv (M⁻¹)</th>
<th>fₒ</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>0.67393</td>
<td>0.52910</td>
</tr>
<tr>
<td>5</td>
<td>2.33</td>
<td>0.69440</td>
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Table 6.1: Fluorescence parameters (Ksv and fₒ) for acrylamide quenching of EhPGDH monitored at pH 7 and 5.

The pH dependent changes in the structural and functional features of the EhPGDH suggest that the protein has undergone significant conformational changes with decrease in pH and an intermediate state stabilized roughly around pH 5. We further characterized the stabilized intermediate to understand the changes in the structural features of the protein associated with acidic pH.

The protein dimer dissociates at pH 5

The perturbations in the tertiary structure of EhPGDH under mild acidic condition tempted us to explore more about the structural features of the protein at pH 5. Gel filtration experiments were performed to study the effect of pH on the molecular dimensions of the protein. Fig. 6.4A shows the pH-dependent SEC profile of EhPGDH on Superdex™ 75 10/300 GL column. At pH 7 the retention volume of EhPGDH was 10.1 mL corresponding to molecular mass of 70 kDa as compared with the standard molecular weight markers. With decrease in pH from 7 to 5, a significant increase in retention volume was observed. The protein was eluted at 11.7 mL which corresponds to molecular mass of ~35 kDa (as compared with the standard molecular weight markers) which is the actual
molecular mass of a single subunit of EhPGDH (Fig. 6.4B). This indicates a pH-dependent decrease in the hydrodynamic radii of the EhPGDH. There can be two possible reasons for such an increase in the retention volume of the protein at pH 5 either, there might be pH dependent subunit dissociation or the dimeric protein has attained a very compact conformation. The later possibility was ruled out by glutaraldehyde mediated chemical cross-linking studies. Fig. 6.4A inset shows a pH dependent cross-linking profile of EhPGDH on a 12 % SDS-PAGE. The cross-linked samples showed protein bands corresponding to the dimer at pH 7 and a monomer at pH 5. The monomeric protein at pH 5 was again re-constituted into dimer by dialysis at pH 7. Thus, the pH dependent dimer dissociation is a completely reversible event. SEC was performed at 10 μM and 100 μM protein concentrations at pH 5 to analyse the concentration dependent protein oligomerization but at both the protein concentrations a single peak with retention volume 11.7 mL was observed corresponding to the enzyme monomer. The results clearly demonstrate that EhPGDH at pH 7 is stabilized as a dimer however at pH 5 the subunit interactions are lost leading to dimer dissociation. The monomer stabilized at pH 5 was almost functionally inactive (as discussed above).

![SEC profile of EhPGDH](Fig. 6.4A)

The protein regained its enzymatic activity in pH-jump experiments between pH 5 and 7 in presence of the cofactor NADH (data not shown). This can be attributed with dimer re-association on pH-jump resulting in the restoration of the enzymatic activity. Thus, both the subunits are essential for the optimal activity of the enzyme. The pH dependent dimer dissociation can be attributed to the protonation of the acidic amino acid residues present at the dimer interface under acidic environment.
(pH 5) resulting in the loss of ionic interaction which stabilizes the subunit assembly. The results suggest that the electrostatic interactions at the subunit interface are primarily responsible for dimer stability.

**Role of electrostatic interactions in subunit assembly**

Intra- and inter-molecular ionic interactions stabilize the tertiary and quaternary structure of the proteins. To understand pH dependent dimer dissociation of the EhPGDH a detailed study of the subunit interface was performed. The protonation of key amino acid residues present at the subunit interface can be associated with dissociation of the enzyme upon lowering of the pH. As estimated from the above discussed data, the apparent pKa, of the dimer dissociation is ~5.0. However, this is not necessarily the pKa of the amino acid residue or residues that are involved in the dissociation. If the pH-dependent dissociation is considered in terms of a linked function phenomena as described by Wyman (Wyman 1948, 1964) for the pH dependent dissociation of haemoglobin, then the data can be interpreted as meaning that monomers of EhPGDH have a higher affinity for protons than to dimers. This indicates that the protonated ionic groups (at pH 5) are dissociation-linked and that their pKa values are different when the enzyme is in the dimer state than when it is in the monomer state. Since no crystal structures of EhPGDH is reported, to identify and analyse the role of key interface residues we developed an *in silico* energetically minimized model of EhPGDH using computational modeling along with a 10ns molecular dynamic simulations. A three-dimensional model of EhPGDH dimer, based on four crystal structure templates of Phosphoglycerate dehydrogenase from (*Escherichia coli*, Protein Data bank code 2PA3, *Mycobacterium tuberculosis*, PDB code 1YGY, *Pyrococcus horikoshii*, PDB code 1WWK and *Homo sapiens*, PDB code 2G76) was generated and subsequently subjected to energy minimization followed by a 10ns molecular dynamics simulation as described under “Material and Methods”. The amino acid residues present at the dimer interface are conserved for PGDH (Ali et al. 2004a). An assessment of the stability of native conformation of the protein can be performed to a close approximation by examining the accessible surface area lost upon dimerization. For EhPGDH the accessible surface area (ASA) calculated with the help of MSMS program for dimer was found to be 27340.56 Å² while the monomer had an ASA of 15374.04 Å². Thus, the percentage area of the monomer lost upon dimerization was ~20 % which accounts to about 3407.52 Å². The results are comparable to a variety of stable dimers (Janin et al. 1988).

**Glu-108 is essential for the dimer stability**: The amino acid residues of the either subunits interact at the dimer interface with the help of salt bridges, hydrogen bonds and hydrophobic contacts (Fig. 6.5A). Furthermore, in order to gain a clear picture about the contribution of each residue at the subunit interface that make significant contributions to the binding free energy associated with dimer formation, the total binding free energy was decomposed into per residue contributions (Fig. 6.5B).
Accordingly, important amino acid residues identified contributing towards dimer formation are Asn104, Glu108, Ile115, Arg119, Arg128, Glu129, Leu130 and Lys133. Interestingly, Glu108 of one subunit forms a salt bridge with Arg119 of the neighboring subunit (Fig. 6.5A). Both the residues are present at a key position on the \(-\)helix that runs along the at the dimer interface. This Glu108 might get protonated along with other acidic amino acid residues on lowering of pH (pH5) which would disrupt the ionic interactions between Glu108 and Arg119 resulting in dimer dissociation. We investigated this possibility by mutating Glu108 with a neutral amino acid residue alanine.

![Fig. 6.5: Analysis of the subunit interface](image)

(A) A snap shot showing the subunit interface of the energy minimized model of EhPGDH after MD simulations. The two subunits are shown in magenta and cyan color. The enclosed boxes show the salt bridge between the Glu108 and Arg119 at the subunit interface highlighted with red color.
E108A mutation of EhPGDH resulted in dimer dissociation at pH 7 as observed by the SEC. The WT-protein on a Superdex™ 75 10/300 GL column showed a retention volume at 10.1 mL which correspond to the molecular weight of 70 kDa corresponding to the protein dimer when with compared to the standard molecular weight markers. However, the E108A mutant was eluted at 11.6 mL at pH 7 which corresponds to a molecular weight of ~36 kDa corresponding to a monomer (Fig.6.6A).

Fig. 6.6: E108A mutant is stabilized as a monomer.
(A) SEC profile of EhPGDH on a Superdex™ 75 10/300 GL column. WT-protein (solid line) and E108A mutant (dashed line).
(B) A logarithmic graph of the elution volume of EhPGDH dimer (WT-protein) and monomer (E108A mutant) plotted against the standard molecular mass markers.

Fig. 6.6B shows a log plot of standard molecular weight markers on which WT-protein showed molecular mass of ~70 kDa and E108A mutant showed molecular mass of ~35 kDa. Similar results were observed with the glutaraldehyde mediated chemical cross-linking studies where no cross-linked protein band corresponding to a dimer was observed for E108A mutant (data not shown). The results clearly explain that loss in the subunit assembly at pH 5 is by virtue of amino acid protonation under acidic conditions. The Glu108 gets protonated at pH 5 in the WT-protein resulting in the loss of the ionic interactions between Glu108 and Arg119 which eventually leads to dimer dissociation.

For an oligomeric protein such as EhPGDH the subunit interface is considered to be important for the structure-function studies. Earlier studies on have shown that subunit interface residues play a subtle role in enzyme catalysis (Breiter et al. 1994). The functional activity of E108A mutant was almost negligible as compared to the WT-protein. Two schools of thought arose surrounding such a structure-function relationship. Harada and Wolfe first proposed the reciprocating compulsory ordered mechanism where each subunit alternates as the “active” and the “helper” subunit, but both
are needed for activity (Harada and Wolfe 1968). This mechanism predicts an inactive monomer, and was corroborated by studies that showed a dramatic reduction of enzymatic activity on dissociation to monomers at low enzyme concentration, at pH 5.0, and in the absence of substrates (Bleile et al. 1977; Wood et al. 1981a; Wood et al. 1981b). The second mechanism introduces equilibrium between 2 conformers, one of which preferentially binds NAD⁺, whereas the other binds NADH (Mullinax et al. 1982). This mechanism suggests presence of an active monomer. Evidence for this idea is exhibited by an immobilized monomeric form of malate dehydrogenase (MDH) (DuVal et al. 1985; Jurgensen et al. 1981) and a hybrid modified form of MDH (Jurgensen et al. 1981; McEvily et al. 1985) having some catalytic function. The EhPGDH at pH 5 as well as the E108A mutant at pH 7 both are stabilized as a monomer with negligible functional activity. This clearly suggests that both subunits are essentially required for optimal enzymatic activity of EhPGDH. Characterization of the monomeric E108A mutant of EhPGDH shows that a relatively small change at the subunit interface can disrupt the extensive subunit-subunit interactions.

6.4 Summary

Mild acidic conditions induce significant alterations in functional and structural features of the EhPGDH (summarized in Fig. 6.7). The protein at pH 7 is stabilized as a functionally active homodimer. The cofactor NADH was present closely associated with the active site of the protein.

\[
\begin{array}{c|c|c|c}
\text{E108A mutant} & \text{WT-protein} & \text{pH 5} & \text{pH 7} \\
\text{Monomer} & \text{Dimer} & \text{Monomer} & \\
(\text{Inactive}) & (\text{Active}) & (\text{Inactive}) & \\
\end{array}
\]

Fig. 6.7: A schematic diagram representing the pH and site directed mutagenesis induced changes in EhPGDH.

Between pH 7 and 5, significant perturbations in the functional and structural features of the protein occur which are marked by changes in the NADH microenvironment and loss of functional activity but the most interesting feature was the loss subunit assembly at pH 5. These events are completely reversible as on dialysis at pH 7 the oligomeric status and functional activity was restored in presence of NADH. The pH dependent loss of the subunit assembly is by virtue of protonation of negatively charged amino acids at the dimer interface. This was proven by the fact that E108A mutant of EhPGDH failed to retain its original dimer configuration and was stabilized as a monomer. The study demonstrates that native dimeric conformation of EhPGDH is governed by a fine balance of electrostatic interactions between the subunits of the protein.