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Purification, preliminary X-ray crystallography and biophysical studies of triose phosphate isomerase-β-globin subunit complex

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ABSTRACT

Triose phosphate isomerase (TIM) is a cytoplasmic enzyme of prime importance in the mammalian glycolytic pathway. It has a major role in the conversion of dihydroxyacetone phosphate into glyceraldehyde-3-phosphate. We have successfully purified a stable complex of TIM with β-globin subunit from the sheep kidney using a simple two-step chromatography procedure. It is seen for the first time that TIM is forming a stable complex with β-globin. The purified protein-protein complex was crystallized and preliminary diffraction data were collected at 2.1 Å resolution. We further studied guanidinium chloride (GdmCl)-induced denaturation of TIM-β-globin complex by monitoring changes in the mean residue ellipticity at 222 nm ($[\theta]_{222}$) and difference absorption coefficient at 406 nm ($\Delta\varepsilon_{406}$) at pH 7.5 and 25 °C. We have observed that GdmCl-induced denaturation is reversible. Coincidence of normalized transition curves of both physical properties ($[\theta]_{222}$ and $\Delta\varepsilon_{406}$) suggests that folding/unfolding of TIM and β-subunit proteins is a two-state process. Denaturation curves of $[\theta]_{222}$ and $\Delta\varepsilon_{406}$ were used to estimate the stability parameters of the protein-protein complex. This is the first report on the isolation, purification, crystallization and biophysical characterization of the naturally occurring complex of TIM with the β-globin subunit.

1. Introduction

Triose phosphate isomerase (TIM) is a key cytoplasmic enzyme. It is a homodimeric protein, and the molecular mass of its subunit is 27 kDa [1]. It is present ubiquitously in almost all kingdoms of life [2,3]. It helps in the carbohydrate metabolism where it catalyzes the glycolytic conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate and vice-versa [4,5]. In human, the gene for TIM is encoded by a single gene located at chromosome 12p13 [6]. TIM has no cofactors and does not require any metal ions for its function. It does not show cooperativity between its subunits. It is important for mammalian life because it checks the deposition of dihydroxyacetone phosphate (DHAP) which is an extremely toxic metabolite. This enzyme is very helpful for acceleration of methylglyoxal-like product which enhances the manufacture of highly developed glycation end products [7–10].

The structure of TIM is well studied. It has a barrel shaped motif found in a large number of other enzymes with broadly different functions having small or no sequence homology [11]. TIM barrel domain is composed of eight alternating α-helices and β-strands called $(\beta/\alpha)_8$ fold which is known as TIM-barrel fold [12–16]. The structural form of α/β-elements of TIM are coiled into a barrel shaped which contains an eight stranded parallel β-sheet surrounded by eight parallel α-helices [17,18]. TIM is completely active when it is in the dimeric form [19] but it forms a tetramer in archaea and some thermophilic bacteria [20]. On the other hand, β-globin subunit is the component of active hemoglobin which is responsible for the oxygen transport.

So far, there is no report available which demonstrates any kind of complex formation between TIM and β-globin. For the first time, we have observed a natural, stable complex formation between TIM and β-globin, which we have successfully purified and crystallized. We have further determined the thermodynamic stability of this novel complex using spectroscopic techniques.

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2. Materials and methods

2.1. Materials

Sheep kidney was purchased from the Slaughter House (Ghazipur, New Delhi). Hi-Trap diethylaminoethanol (DEAE–FF) and Superdex 200 pg gel filtration columns were provided by GE Healthcare (USA). Gel electrophoresis system and reagents were purchased from Bio-Rad Laboratories (USA). Guanidinium chloride (GdmCl) was an ultrapure sample purchased from MP Biomedicals. Other reagents of analytical grade were provided by Merck, Sigma-Aldrich, and local suppliers.

2.2. Protein isolation

Sheep kidneys were chopped in small pieces, washed with distilled water and soak in phosphate buffer saline (PBS) at pH 7.4. After complete removal of blood, pieces of kidney in buffer (pH 7.4) containing 1 mM EDTA, 1 mM PMSF and 1 m DTT were crushed with the help of blender. We have used 1.6 ml of phosphate buffer for 1 g of wet tissue. All purification steps were performed at 4 °C. Two layers of muslin cloth were used for filtration of homogenate which was kept for overnight stirring at 4 °C. This filtered homogenate was centrifuged at 12,000 rpm for 20 min, subsequently pellet was discarded and supernatant was collected for the next purification step to ammonium sulphate precipitation. The supernatant was saturated by 30%, 60% and 90% ammonium sulphate. The precipitate obtained after 90% saturation was dissolved in 10 mM Tris–HCl buffer of pH 8.0 and dialyzed in the same buffer before proceeding to the next purification step.

2.3. Gel chromatography

The dialyzed sample was filtered through 0.22 μm Millipore, loaded on the weak anion Hi-Trap DEAE FF (1 ml, 7 mm × 25 mm) column which was connected to the Akta purifier. 10 mM Tris–HCl buffer (pH 8.0) was used to pre-equilibrate this column. Protein was subjected to the column at a constant flow rate of 0.5 ml/min, and the unbound proteins were washed with the same buffer. We used a gradient from 0 to 1.0 M NaCl for the elution of the bound proteins. The first major peak eluted at 0.1 M of NaCl was pooled and concentrated. 0.5 ml of concentrated and filtered protein was passed through the gel permeation Superdex (200 pg) column, pre-equilibrated with 10 mM Tris–HCl (pH 8.0) and connected to the Akta purifier. Protein was eluted at a rate was 0.5 ml/min. For the analysis of elution profile, Unicorn manager Version 5.31 software was used.

2.4. Gel electrophoresis

SDS-PAGE was run using a previously described protocol [21] to check the purity of the protein. SDS-PAGE was done in a slab gel assembly with 15% resolving gel and 5% stacking gel. For gel staining Coomassie brilliant blue (G-250) was used. Protein markers of molecular mass ranging from 180 to 10 kDa were used for the estimation of molecular mass. Native PAGE was also run using the same procedure with slight modifications.

2.5. Mass spectrometry

The bands of TIM and β-globin subunit were cut with the help of sterile blade from the SDS-PAGE to get peptide mass fingerprints using a method described elsewhere [22–24]. A MASCOT (version 1.1.2.0) distiller software was used to calculate the observed mass spectra, peak area versus mass/electric charge (m/z) of monoisotopic ions.

2.6. Crystallization and data collection

The protein-protein complex was crystallized by using hanging drop vapor diffusion method at a concentration of 15 mg ml^{-1}. The crystals were produced in a week using 10 Mm Tris–HCl at pH 8.0 and 20% PEG 5000. The crystal was flash-cooled in liquid nitrogen stream before the data collection. X-ray diffraction data were collected at 278 K using X-ray generator (Rigaku FR-E+ Super Bright micro focus rotating anode). The diffraction data were collected on R-AXIS IV^{++} detector at 100 K. A data set was collected using 70 frames with a one degree oscillation angle. During the complete data collection, crystal was stable in the beam. The data obtained were indexed and integrated using DENZO/SCALEPACK data processing software [25].

2.7. Circular dichroism (CD) measurements

The far-UV CD spectrum (250–200 nm) of TIM-β-globin complex was measured in Jasco-1500 CD machine which was equipped with Peltier-type temperature controller (PTC-517). Each CD spectrum was corrected for contribution of the blank solution and run with 5 accumulations at 25 ± 0.1 °C. The protein concentration used was 0.3 mg ml^{-1}. The CD values were converted into concentration-independent parameter, i.e., mean residue ellipticity, $[\theta]_\lambda$ (deg cm^2 dmol^{-1}) using the relation,

$$[\theta]_\lambda = M_o\theta_\lambda/10lc \quad (1)$$

where M_o is the mean residue weight of the protein, θ_λ is the observed ellipticity in millidegrees, c is the concentration in mg ml^{-1}, and l is the path length of the cell in cm.

2.8. Measurements of absorption spectra

The absorption spectra (550–240 nm) of TIM-β-globin subunit complex were measured in Jasco (V-660) UV/visible spectrophotometer with a 1 cm path length cell at 25 ± 1 °C. This equipment was connected to a Peltier-type temperature controller (ETCS-761). For GdmCl-induced denaturation studies, TIM-β-globin samples were prepared in different molar concentrations of GdmCl at 25 ± 1 °C and incubated for overnight. The TIM-β-globin concentration of 0.3 mg ml^{-1} was used for each measurement. For the concentration independent-parameter, the change in molar absorption coefficient ($\Delta\varepsilon$) was calculated from absorbance data and plotted as a function of GdmCl concentration.

2.9. Analysis of transition curve

By plotting each of the spectral properties ($[\theta]_{222}$ and $\Delta\varepsilon_{406}$) versus [GdmCl], the molar concentration of GdmCl, we obtained denaturation curves. Both the transition curves were analyzed for stability parameters $\Delta G_D{}^0$, the value of ΔG_D (change in Gibbs free energy associated with the denaturation process, native (N) state ↔ denatured (D) state) in the absence of GdmCl; m, the slope ($\partial\Delta G_D/\partial$[GdmCl]); and C_m (=$\Delta G_D{}^0/m$), the denaturation curve midpoint where $\Delta G_D = 0$. A non-linear least-squares method was used to fit the entire denaturation curve obtained at constant temperature and pH according to the equation [26]

$$y(g) = y_N(g) + y_D(g)_* \mathrm{Exp}[-(\Delta G_D{}^0 + m[g])/RT]/ (1 + \mathrm{Exp}[-(\Delta G_D{}^0 + m[g])/RT]) \quad (2)$$

where y is the optical property used to follow denaturation curve, y_N and y_D are the properties of the native and denatured states of