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Mass spectrometric analysis of dimer-disrupting mutations in *Plasmodium* triosephosphate isomerase

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ABSTRACT

Electrospray ionization mass spectrometry (ESI MS) under nanospray conditions has been used to examine the effects of mutation at two key dimer interface residues, Gln (Q) 64 and Thr (T) 75, in *Plasmodium falciparum* triosephosphate isomerase. Both residues participate in an intricate network of intra- and intersubunit hydrogen bonds. The gas phase distributions of dimeric and monomeric protein species have been examined for the wild type enzyme (TWT) and three mutants, Q64N, Q64E, and T75S, under a wide range of collision energies (40–160 eV). The results established the order of dimer stability as TWT > T75S > Q64E ~ Q64N. The mutational effects on dimer stability are in good agreement with the previously reported estimates, based on the concentration dependence of enzyme activity. Additional experiments in solution, using inhibition of activity by a synthetic dimer interface peptide, further support the broad agreement between gas phase and solution studies.

Introduction

The glycolytic enzyme triosephosphate isomerase (TIM) functions as an obligate dimer in bacteria and eukaryotes [1] and a tetramer in archaea [2]. The catalytic site consists of four completely conserved residues, K12, H95, E97, and E165, which are all located on one subunit [3]. The precise positioning of the active site residues is determined by important hydrogen bond contacts across the subunit interface. Engineered monomers have been shown to be fully folded into the characteristic α_8/β_8 TIM barrel structure, but are over a 1000 fold less active in mediating the proton transfer reaction, which interconverts the two triosephosphates formed during glycolysis [4]. The availability of a large number of TIM sequences from diverse organisms has permitted the identification of sequence positions that are completely conserved or at which extremely limited amino acid diversity is observed. Two positions, residue 75, where Thr is completely conserved, and residue 64, at which only Gln (Q) or Glu (E) is observed, participate in hydrogen bonds across the subunit interface. Fig. 1 shows a view of the interactions involving Q64 and T75 in the native enzyme structure. A detailed kinetic and structural analysis of site-specific mutants at these two positions has established that subunit dissociation is facilitated in the Gln64Glu (Q64E) and Gln64Asn (Q64N) mutants, whereas replacement of Thr (T) 75 by Ser (S), Val (V), Cys (C), and Asn (N) does not significantly affect the dimeric nature of the enzyme in solution [5].

We describe in this report nano-ESI MS studies of site-specific mutants constructed using *Plasmodium falciparum* TIM (*Pf*TIM) as a template, which addresses the effect of residue replacements at positions 64 and 75. Nanoelectrospray ionization mass spectrometry (Nano-ESI MS) has emerged as a powerful method for probing the nature of oligomeric protein structures, with gentle ionization and desolvation protocols permitting characterization of protein–protein complexes, which resemble those in solution [6–9]. The population of monomeric species in solution has been used to develop a synthetic interface peptide as an inhibitor of subunit association.

Materials and methods

Protein purification

The procedure for the purification of *Pf* TIM wild type was similar to that in previous studies [10]. All the mutants were purified by a modification of this procedure that omits an ion-

Abbreviations: ESI MS, Electrospray ionization mass spectrometry; LC, Liquid chromatography; *Pf*, *Plasmodium falciparum*; QTOF, Quadrupole time-of-flight; TIM, Triosephosphate isomerase; TWT, *Pf*TIM wild type.

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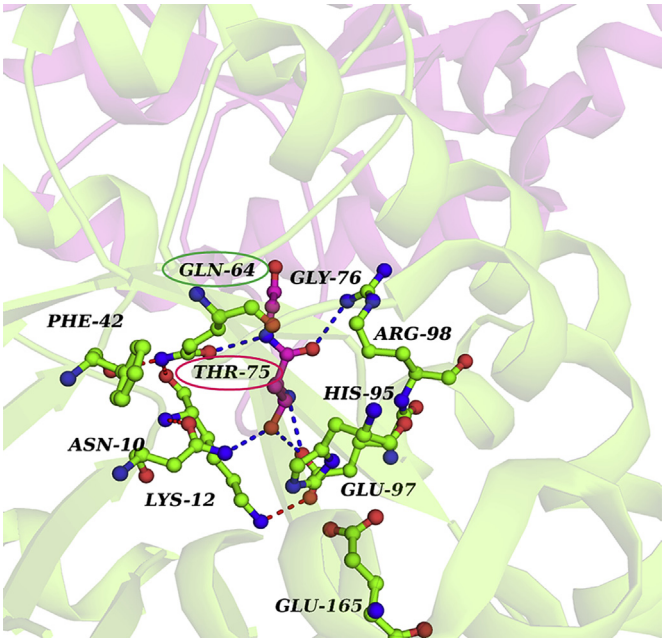

Fig.1. The network of conserved inter- and intrasubunit hydrogen bonds at the dimer interface of *Pf*TIM wild type (PDB: 1O5X). Positions of the residues of interest (Gln 64 and Thr 75) are circled.

exchange chromatography step due to the poor solubility of the mutant proteins under the conditions used. The bacterial cells containing the proteins were disrupted by sonication. The soluble fractions were subjected to 40% and 80% ammonium sulfate precipitation. Purified proteins were obtained in the supernatant of the 80% ammonium sulfate precipitation. Prolonged dialysis was carried out for the removal of the excess salt, against 20 mM Tris–HCl (pH 8.0) buffer. The purity of the wild type and mutants were checked by SDS-PAGE and LC-ESI MS. The molecular masses obtained by LC-ESI correspond well to the calculated masses of the mutant enzymes, $M_{observed}$ ($M_{calculated}$), Da: 27831.3 (27831.5) TWT; 27818.2 (27817.8) T75S; 27817.8 (27817.5) Q64N; and 27833.3 (27832.5) Q64E [5]. Protein concentrations were determined by the Bradford assay [11], using BSA as a standard.

Mass spectrometry

All spectra were acquired in an ESI-QTOF Maxis impact (Bruker Daltonics, Bremen, Germany) mass spectrometer, equipped with a nanoelectrospray source. Samples for mass spectrometry were prepared by diluting the concentrated proteins to a concentration of 6 μM (concentrations were calculated, using the monomeric molecular mass of the proteins) in 10 mM ammonium bicarbonate buffer at pH ~7.4. This pH value corresponds to that measured in the bulk solution before infusion into the mass spectrometer. Samples were injected through a picotip emitter silica tip (nanoflow probe silica tips, inner diameter 10 μm, New Objectives, Woburn, MA, USA). A syringe pump and needle connected to a 5 μl loop were used for sample infusion into the spray chamber (830 nL/min). External calibration was done using caesium iodide in the range of *m*/*z* 500–6000. The mass spectrometer was operated in the positive ion mode.

For the detection of the TIM dimer in the nano-ESI mode, a capillary voltage of 1.2–1.6 kV and a source block temperature of 40–45 °C (dry N_2) were maintained to preserve noncovalent interactions. In-source energy (isCID) (40–160 eV) and collision-induced dissociation energy (CID) (40–160 eV) were optimized to acquire the wild type spectra. The tubing was flushed with water before injection of the sample. For the wild type enzyme, spectra were acquired at different values of in-source CID and at different collision energy values in the quadrupole stage. Fig. S1 (Supplementary Data) shows wild type nano-ESI spectra acquired, using different isCID and at a constant CID. Instrumental parameters, transfer time (160–200 μs), and prepulse storage time (20–45 μs) were optimized for detection of wild type protein dimers. Protein spectra were acquired at different CID energies, keeping the in-source CID and the collision gas temperature fixed.

Data acquisition was carried out using Bruker data analysis software (version 4.1). The mass spectral data are presented as raw data, on an *m*/*z* scale, with minimal smoothening by the Savitzky–Golay method. Protein masses were computed from the charge state distributions using the in-built deconvolution algorithm.

Solid phase synthesis of the TIM interface peptide (residues 61–87)

The 27-residue peptide TGIQNVSKFGNGSYTGEVSAEIAKDLN, corresponding to residues 61–87 of *Pf*TIM, was synthesized by standard Fmoc solid phase peptide protocols using a PS3 peptide synthesizer (Protein Technologies, Arizona, USA) on a target scale of 0.1 m equiv, corresponding to 300 mg of resin (loading capacity 0.63 mmol/g). The side chains Asp, Glu, Ser, and Thr were protected by tertiary butyl groups, while the side chains of Asn and Gln were protected by a trityl group and that of Lys by a tert-butoxy carbonyl group. The peptide was simultaneously cleaved off the resin and deprotected using reagent K [TFA/phenol/anisole/water/EDTA (82.5:5:5:5:2.5)] after the synthesis. After complete deprotection, the resin was filtered off, trifluoroacetic acid (TFA) was removed by evaporation in vacuo, and the peptide was precipitated with diethyl ether. The precipitate was repeatedly washed with ether and the crude peptide was isolated after centrifugation by decanting the supernatant. The crude peptide was purified by HPLC over a Phenomenex C18 column (9.4 mm × 250 mm, 5–10 μm particle size) using an acetonitrile/H_2O/0.1% TFA solvent system. The flow rate was maintained at 1.0 mL min^{-1}, following a linear gradient of 3–100% acetonitrile over 25 min, and the fractions were detected at 226 nm. The purified synthetic peptide was characterized using ESI MS: $[M+H]^+_{calculated} = 2800.0$ Da, $[M+H]^+_{observed} = 2799.5$ Da.

Enzyme assays

Activity measurements were carried out according to the procedure of Plaut and Knowles (1972) [12] on a Cary UV double-beam spectrophotometer at room temperature. The cuvette contained 100 mM triethanolamine buffer (TEA) at pH 7.6, 5 mM EDTA, 0.1 mM NADH, 20 μg/ml coupling enzyme (α-glycerophosphate dehydrogenase), and 2 mM D-glyceraldehyde-3-phosphate (substrate). Enzyme activity was monitored by the decrease in absorbance at 340 nm. To monitor the effect of the interface peptide on the dimer monomer equilibrium, ~200 nM of *Pf*TIM wild type enzyme was preincubated at three different concentrations of the peptide at the ratios 1:10 (2 μM peptide), 1:100 (20 μM peptide), and 1:1000 (200 μM peptide) for 1 h and 3.5 h at 25 °C. Dilution of 7.5 μl of this solution to 500 μl in the final reaction mixture yielded a final enzyme concentration of 2.9 nM. Similarly, the inhibitory effects of the designed peptide were tested on the two dimer interface mutants Q64N and Q64E using concentrations of 28 nM and 42 nM protein, respectively, in the reaction mixture. The control sample had the same enzyme concentration with no added peptide.