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Dynamic Requirements for a Functional Protein Hinge

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The enzyme triosephosphate isomerase (TIM) is a model of catalytic efficiency. The 11 residue loop 6 at the TIM active site plays a major role in this enzymatic prowess. The loop moves between open and closed states, which facilitate substrate access and catalysis, respectively. The N and C-terminal hinges of loop 6 control this motion. Here, we detail flexibility requirements for hinges in a comparative solution NMR study of wild-type (WT) TIM and a quintuple mutant (PGG/GGG). The latter contained glycine substitutions in the N-terminal hinge at Val167 and Trp168, which follow the essential Pro166, and in the C-terminal hinge at Lys174, Thr175, and Ala176. Previous work demonstrated that PGG/GGG has a tenfold higher K_m value and 10^3-fold reduced k_{cat} relative to WT with either d-glyceraldehyde 3-phosphate or dihyrdroxyacetone phosphate as substrate. Our NMR results explain this in terms of altered loop-6 dynamics in PGG/GGG. In the mutant, loop 6 exhibits conformational heterogeneity with corresponding motional rates $<750\ s^{-1}$ that are an order of magnitude slower than the natural WT loop 6 motion. At the same time, nanosecond timescale motions of loop 6 are greatly enhanced in the mutant relative to WT. These differences from WT behavior occur in both apo PGG/GGG and in the form bound to the reaction-intermediate analog, 2-phosphoglycolate (2-PGA). In addition, as indicated by ^1H, ^{15}N and ^{13}CO chemical-shifts, the glycine substitutions diminished the enzyme's response to ligand, and induced structural perturbations in apo and 2-PGA-bound forms of TIM that are atypical of WT. These data show that PGG/GGG exists in multiple conformations that are not fully competent for ligand binding or catalysis. These experiments elucidate an important principle of catalytic hinge design in proteins: structural rigidity is essential for focused motional freedom of active-site loops.

Keywords: enzyme catalysis; loop motion; protein hinge; NMR spectroscopy; protein dynamics

Introduction

Enzymes make extensive use of conformational changes throughout their catalytic cycle that are, in many cases, essential to their function. Triosephosphate isomerase (TIM, EC 5.3.1.1) is an important case in which motion plays a significant role in the rate-limiting catalytic step. TIM is a very efficient and faithful catalyst of the interconversion (Scheme 1) between dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP), enabling isomerization near the diffusion-limited rate,[1] while limiting formation of the toxic side product, methylglyoxal, to only one molecule per 10^5 catalytic cycles.[2] A critical aspect of TIM catalysis is the participation of a highly conserved active-site Ω loop,

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Abbreviations used: TIM, triosephosphate isomerase; cTIM, chicken TIM; DHAP, dihydroxyacetone phosphate; GAP, [D]-glyceraldehyde 3-phosphate; 2-PGA, 2-phosphoglycolate; WT, wild-type; PGG, P166/V167G/W168G; GGG, K174G/T175G/A176G; PGG/GGG, (PGG+GGG); HSQC, heteronuclear single-quantum coherence; TROSY, transverse relaxation optimized spectroscopy; ssNOE, steady-state nuclear Overhauser effect; rmsd, root-mean-square deviation.

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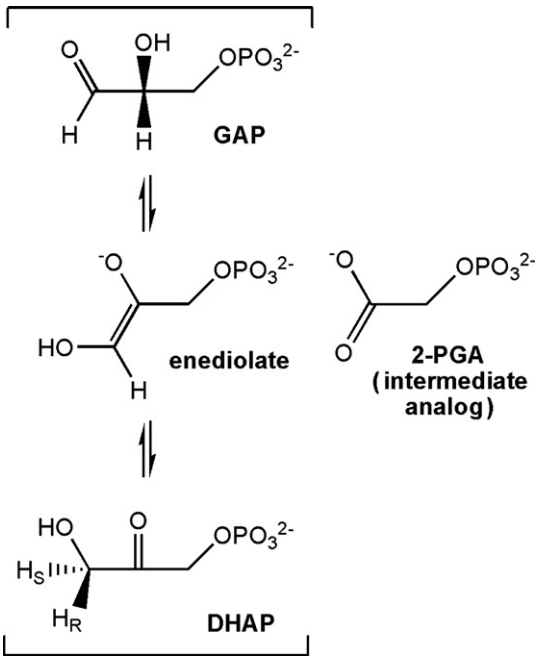


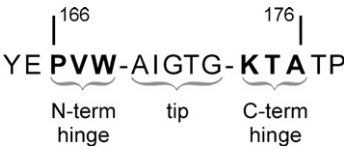
Scheme 1. TIM-catalyzed reaction scheme (bracketed) with enediolate intermediate and adjacent structure of the reaction intermediate analog, 2-phosphoglycolate (2-PGA) at the right.

the 11 residue loop 6, which consists of three residue N and C-terminal hinges and an intervening five residue tip (Scheme 2). Ω loops typically reside on the surface of proteins and consist of 6–20 amino acid residues with a short distance (≤10 Å) between the N and C-terminal residues, yielding a resemblance to the Greek letter Ω.[3,4]

In TIM, loop 6 plays a functional role *via* its motion between two major conformational states: open and closed (Figure 1). In the open conformation, the substrate has ready access to both the active site and the bulk solvent. The closed conformation is observed in X-ray crystallographic studies in which ligand is bound in the TIM active site. Closure of loop 6 involves an approximately 7 Å movement at its tip (C^{α} of Thr172). This closed form is stabilized by hydrogen bonds between the amide NH of loop 6 residue Ala176 and the hydroxyl group of Tyr208 within loop 7 (residues 208–211) and between the Ala176 carbonyl group and the Ser211 hydroxyl group†. Notably, loop 6 closure is accompanied by significant structural changes in loop 7 that allow repositioning of the carboxylate group of Glu165, which is the catalytic base, into its functionally competent position.[5–7] In addition, the central five residues of loop 6 have been shown to be important for preventing unwanted production of methylglyoxal,[2] yet the only direct contact of loop 6 with the ligand is a single hydrogen bond between the ligand phosphate group and the backbone NH of Gly171. This stabilization of an intermediate state and the possible coordination of loop 6 motion with that of loop 7 and Glu165 facilitate the enzymatic reaction, and physically allow passage of the ligand between the active site and solvent. Thus, motion of loop 6 is essential to overall function in TIM.

Several lines of evidence support the notion that loop 6 motion is limiting to the reaction rate in the direction of the DHAP to GAP interconversion,[1] each placing loop motion of the order of 10^3–10^4 s^{-1} in yeast (*Saccharomyces cerevisiae*) TIM. Computational studies have long suggested that loop 6 moves on a microsecond timescale with rate-limiting effect.[8,9] Solid-state NMR relaxation studies[10,11] of ^{2}H-labeled Trp168 and solution-state NMR studies[12] of ^{19}F-labeled Trp168 also indicate that loop 6 moves at a rate of 10^4 s^{-1} and in a manner that is likely rate-limiting to catalysis. Furthermore, these rates were confirmed by temperature-jump relaxation spectroscopy utilizing Trp168 fluorescence,[13] and supported by a study revealing that conformational motion on this timescale enhances ^{15}N spin-relaxation rates within loop 6 and other sites vicinal to it.[14,15] In addition, the solid-state[10] and solution-state[14,15] NMR experiments indicate that loop 6 moves in both apo and bound enzyme forms and, therefore, that loop motion is not ligand-gated.

The interconversion between opened and closed conformations of loop 6 is a rigid-body motion in which only residues in the N and C-terminal hinges experience changes in backbone dihedral angles.[16,17] Hydrogen bonds involving loop 6 residues are another prominent feature of the open-close motion. Upon closure, the amide NH of Gly171 in the tip of the loop comes within 2.8 Å of the O3 oxygen atom in the substrate phosphate, whereas the NH of Ala176 in the C-terminal hinge forms a critical hydrogen bond with the Tyr208 hydroxyl in loop 7.[8,18,19] Upon closure, tight intra-loop hydrogen bonding also occurs between the amide groups of Ala169 and Ile170 in the loop 6 center and carbonyl groups of Pro166 and Val167 in the N-terminal hinge, respectively. Not surprisingly, residues in loop 6 are highly conserved in over 130 TIM sequences as described,[20] and as investigated by genetic,[21,22] kinetic[23,24] and crystallographic[20] means. In the loop 6 center, sequence conservation is driven by a structure that: (1) encapsulates the active site during catalysis; (2) facilitates the Gly171 backbone hydrogen bond to substrate and the intra-enzyme hydrogen bonds of Ala169 and Ile170; and



Scheme 2. Highly conserved loop 6 sequence in TIM, here labeled for amino acid residues 166–176 found in chicken (*Gallus gallus*) TIM. Nomenclature used in the text assigns N1, N2, N3 to N-terminal hinge residues 166–168 and C1, C2, C3 to C-terminal hinge residues 174–176, respectively.

† Amino acid numbering follows the convention for chicken (*Gallus gallus*) TIM.