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Structural analysis of substrate recognition by glucose isomerase in Mn^{2+} binding mode at M2 site in *S. rubiginosus*

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ABSTRACT

Glucose isomerase (GI) catalyzes the reversible enzymatic isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively. This is one of the most important enzymes in the production of high-fructose corn syrup (HFCS) and biofuel. We recently determined the crystal structure of GI from *S. rubiginosus* (SruGI) complexed with a xylitol inhibitor in one metal binding mode. Although we assessed inhibitor binding at the M1 site, the metal binding at the M2 site and the substrate recognition mechanism for SruGI remains the unclear. Here, we report the crystal structure of the two metal binding modes of SruGI and its complex with glucose. This study provides a snapshot of metal binding at the SruGI M2 site in the presence of Mn^{2+}, but not in the presence of Mg^{2+}. Metal binding at the M2 site elicits a configuration change at the M1 site. Glucose molecule can only bind to the M1 site in presence of Mn^{2+} at the M2 site. Glucose and Mn^{2+} at the M2 site were bridged by water molecules using a hydrogen bonding network. The metal binding geometry of the M2 site indicates a distorted octahedral coordination with an angle of 55–110°, whereas the M1 site has a relatively stable octahedral coordination with an angle of 85–95°. We suggest a two-step sequential process for SruGI substrate recognition, in Mn^{2+} binding mode, at the M2 site. Our results provide a better understanding of the molecular role of the M2 site in GI substrate recognition.

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1. Introduction

Glucose isomerase (GI, or xylose isomerase; EC 5.3.1.5) catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively [1]. This protein is a crucial enzyme in sugar metabolism, as the interconversion of xylose to xylulose serves a nutritional requirement [1], found in bacteria, fungi, and plants [2,3]. The isomerization of glucose to fructose is an industrially significant reaction used to produce the sweetener high-fructose corn syrup (HFCS) [4,5]. Moreover, the bioconversion of hemicellulose to ethanol is important for the production of biofuel [6,7].

GI consists of two structural domains with a TIM-barrel domain and a loop that form small interconnected helices, which form a tetrameric quaternary structure [8]. Two metal binding sites, on the active site, are located on the TIM-barrel fold and act as a substrate binding (M1) and catalytic (M2) site to promote hydride shifts [9]. GI requires a divalent cation, such as Mg^{2+}, Mn^{2+}, or Co^{2+}, for catalytic activity in substrate isomerization [10]. Mg^{2+} or Mn^{2+} acts as an activator and Co^{2+} acts as a protein stabilizer to maintain the ordered conformation [1]. The combination of these divalent ions maximizes isomerase activity [11], whereas Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} inhibit isomerase activity [1].

Several GI crystal structures have been reported as complexed with substrates, analogues, or inhibitors [12–14]. Previous work has described substrate-binding moieties. However, the structural analysis of enzymatic processes occurring between metal binding sites and their substrates has not been fully described. We recently reported a GI crystal structure from *Streptomyces rubiginosus* (SruGI), in complex with the inhibitor xylitol, which exhibits metal binding to the M1 site [13]. This structure shows three xylitol oxygen atoms bound to the metal at the M1 site, in the absence of metal ions at the M2 site. While we performed an extended study of

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GI, for substrate recognition, we noticed two questions that have not been structurally studied previously. First, previous SruGI structures exhibits one metal binding mode, despite SruGI crystallized in crystallization solution containing 100 mM $MgCl_2$ [13]. We assume that Mg^{2+} is not stably bound to the M2 site or binding occurs at a very low occupancy, however there is no structural evidence. Second, we questioned whether the glucose corresponding to the substrate could bind to M1 irrespective of the metal bound at the M2 site. Therefore, the mechanisms of metal binding at the M2 site and SruGI substrate recognition were assessed.

To address these questions, we determined the crystal structures of SruGI in a solution containing Mn^{2+}, glucose, or Mn^{2+}-glucose. Our results show that Mn^{2+} was bound to the M2 site with a distorted octahedral coordination caused by conserved residues and water molecules. Glucose molecules do not bind to the M1 site when there is no metal bound at the M2 site; whereas, they bind to the M1 site when Mn^{2+} is bound at the M2 site. Based on the crystallographic results, we suggest that M2 metal and substrates should be sequentially bound to the active site during substrate isomerization of GI. Our results provide a better understanding of the GI substrate recognition mechanism when in the mode of Mn^{2+} binding at the M2 site.

2. Materials and methods

2.1. Protein preparation and crystallization

The protein sample preparation and crystallization of SruGI were performed as previously reported [13]. SruGI was purchased from Hampton research. Buffer was employed as the protein solution and changed to a solution containing 10 mM HEPES, pH 7.5, and 1 mM $MgCl_2$ using Amicon Ultra Centrifugal Filters (Merck Millipore). For crystallization, protein solution was concentrated to 20–30 mg/mL. The protein were prepared and stored at 4 °C. SruGI crystals were obtained by the hanging drop vapor diffusion method at 20 °C. Crystals were obtained by mixing equal amounts of protein solution and reservoir solution containing 10–20 (v/v) PEG 400 and 100 mM $MgCl_2$.

2.2. X-ray diffraction data collection

X-ray Diffraction data for all SruGI crystals were collected at Beamline 7 A at the Pohang Accelerator Laboratory (PLS-II, Pohang) using a Quantum 270 CCD detector (ADSC) [15]. Before crystals were flash frozen in a N_2 stream, the crystals were soaked for 1 min in the following three cryoprotectant solutions containing the 10% (v/v) PEG400, 100 mM $MgCl_2$, and 25% (v/v) ethylene glycol supplemented with (i) 10 mM Mn^{2+}, (ii) 2 mM glucose, or (iii) 10 mM Mn^{2+} and 2 mM glucose. The diffraction datasets were indexed, integrated, and scaled with HKL2000 [16]. A summary of the statistics for data processing is provided in Table 1.

2.3. Structure determination

The structures were solved by molecular replacement with MOLREP [17] using the crystal structure of SruGI in one metal binding mode (PDB code: 5Y4I) as the template [13]. Molecules were manually built using the program COOT [18]. The structure was refined with the Phenix.refine refinement setting in Phenix GUI [19]. The geometry of the models was analyzed with MolProbity [20]. All molecular structures shown were generated with PyMOL (http://pymol.org/) and LigPlot+ [21]. A summary of the statistics for refinement is provided in Table 1. The coordinates and structural factors have been deposited in the Protein Data Bank under the accession codes 5ZYC (SruGI soaked with Mn^{2+}), 5ZYD (SruGI oaked with Glucose), and 5ZYE (SruGI soaked with Mn^{2+} and Glucose).

3. Results and discussion

3.1. Overall structure

To better understand substrate recognition, SruGI crystals were soaked in cryoprotectant solution containing Mn^{2+}, glucose, or Mn^{2+}-glucose. All SruGI crystal structures were refined at a high resolution in the range of 1.40–1.75 Å (Table 1). These crystals belonged to space group I222 and contained one subunit in the asymmetric unit, which forms tetrameric quaternary architecture with symmetry equivalent molecules. The details of the SruGI structure has been previously reported [13]. Briefly, the overall SruGI structures are very similar to those previously deposited. The SruGI structure contains a larger catalytic domain (residues 1–377) with a TIM-barrel fold and a C-terminal domain (residues 378–437) forming an extended tail containing three α-helices (Fig. 1A). Two metal binding (M1 and M2) sites are located in the internal space of the TIM-barrel as a catalytic domain. Although the SruGI crystals were soaked in diverse molecules, such as Mn^{2+} or glucose, each subunit is highly similar to the r.m.s.deviation values of 0.11–0.16 Å on Cα atoms. To assess whether small molecules, such as metal and glucose, affect a conformational change in the secondary structures of the active site, the temperature B-factor of GI was analyzed. The amino acid backbone at the substrate binding position, and the metal on top of the TIM-barrel, showed high rigidity in all structures (Fig. 1B). As stated, in all crystal structures, the loop between the β1-stand and α2-helix shows the highest flexibility, and this area contains the substrate entrance (Fig. 1B). In data for SruGI crystals soaked in Mn^{2+} or Mn^{2+}-glucose, electron density maps are well defined for the metal and substrate, whereas electron maps for glucose were not observed in SruGI crystals soaked in glucose (Fig. 1C–E). All metal and substrate binding were verified by stimulated composite omit map.

3.2. Mn^{2+} at the M2 site

We previously determined GI structures in which only one metal exists at the M1 site, despite crystallization in reservoir solution containing 100 mM $MgCl_2$ [13]. Instead of metal ions, a water molecule is present at M2 site (Fig. 2A). Here we used the same crystallization batch as employed for the one metal SruGI binding mode (denoted as the M1 mode). Since the high Mg^{2+} concentration does not exhibit binding at the M2 site, we attempted to bind the Mn^{2+} at the M2 site, as Mn^{2+} is combined with Mg^{2+} for use as an activator of GI activity [1,4]. Moreover, SruGI displacement studies, assessing visible absorbance, showed that Mn^{2+} has a higher affinity for the metal binding at the M2 site than that of Mg^{2+} [22]. Thus, to visualize the SruGI two metal binding mode (denote as M1-M2 mode), we soaked the SruGI crystal in a cryoprotectant solution containing Mn^{2+} to confirm whether Mn^{2+} binds to the SruGI M2 site provisionally. We observed a strong Fo-Fc (>7 σ) electron density map as metal at the SruGI M2 site (Fig. 1C). The temperature factors of Mn^{2+} (11.70 $Å^2$) at the M2 site were lower than that of the whole protein atom (14.92 $Å^2$), indicating that metal ion rigidly interacted with the SruGI M2 site. This Mn^{2+} is coordinated by Glu217 (2.07 Å), His220 (2.56 Å), Asp255 (2.42 and 2.21 Å for two Oδ atoms bidentate), Asp257 (2.41 Å), and water molecules (Fig. 2B). Among these interactions, the carboxylate group of Glu217 interacts with both metal ions at the M1 and M2 sites (Fig. 2C). Thus, the presence of Mn^{2+} at the M2 site could affect the M1 site configuration. In the M1 mode, metal ions interact with Glu181, Glu217, Asp245, and Asp287 at distances of 2.07 Å, 1.98 Å,

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