Crystal Structures of N-Acetylglucosamine-phosphate Mutase, a Member of the α-D-Phosphohexomutase Superfamily, and Its Substrate and Product Complexes*

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N-Acetylglucosamine-phosphate mutase (AGM1) is an essential enzyme in the synthetic process of UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is a UDP sugar that serves as a biosynthetic precursor of glycopolysaccharides, and the cell wall of bacteria. Thus, a specific inhibitor of AGM1 from pathogenetic fungi could be a new candidate for an antifungal reagent that inhibits cell wall synthesis. AGM1 catalyzes the conversion of N-acetylglucosamine 6-phosphate (GlcNAc-6-P) into N-acetylglucosamine 1-phosphate (GlcNAc-1-P). This enzyme is a member of the α-D-phosphohexomutase superfamily, which catalyzes the intramolecular phosphoryl transfer of sugar substrates. Here we report the crystal structures of AGM1 from Candida albicans for the first time, both in the apoform and in the complex forms with the substrate and the product, and discuss its catalytic mechanism. The structure of AGM1 consists of four domains, of which three domains have essentially the same fold. The overall structure is similar to those of phosphohexomutases; however, there are two additional β-strands in domain 4, and a circular permutation occurs in domain 1. The catalytic cleft is formed by four loops from each domain. The N-acetyl group of the substrate is recognized by Val-370 and Asn-389 in domain 3, from which the substrate specificity arises. By comparing the substrate and product complexes, it is suggested that the substrate rotates about 180° on the axis linking C-4 and the midpoint of the C-5—O-5 bond in the reaction.

UDP-N-acetylglucosamine (UDP-GlcNAc) is a UDP sugar that is synthesized from fructose 6-phosphate (Fru-6-P) supplied from the glycolytic pathway (1–4). UDP-GlcNAc is an essential metabolite that serves as a biosynthetic precursor of glycoproteins, mucopolysaccharides, and the cell wall of bacteria. Recently, it has been found that aberrant modification of O-linked N-acetylglucosamine, which is synthesized from UDP-GlcNAc, is closely linked with diabetes, cancer, and Alzheimer disease (5). The biosynthetic mechanisms of UDP-GlcNAc in prokaryotes and eukaryotes are very similar, but there are significant differences between them (6) (Fig. 1). In eukaryotes, UDP-GlcNAc is synthesized from Fru-6-P by four successive reactions (1–4): (i) the conversion of Fru-6-P into glucosamine 6-phosphate (GlcN-6-P) by glutamine-Fru-6-P amidotransferase, (ii) the acetylation of GlcN-6-P into N-acetylglucosamine 6-phosphate (GlcNAc-6-P) by GlcN-phosphate acetyltransferase, (iii) the interconversion of GlcNAc-6-P and N-acetylglucosamine 1-phosphate (GlcNAc-1-P) by N-acetylglucosamine-phosphate mutase (AGM1),2 and (iv) the pyrophosphorylation of GlcNAc-1-P into UDP-GlcNAc by UDP-GlcNAc pyrophosphorylase (UAP1). In prokaryotes, intramolecular phosphoryl transfer (step iii) occurs before acetylation (step ii). Especially in Escherichia coli, acetylation and subsequent uridylation are catalyzed by a bifunctional enzyme, GlmU (7, 8).

AGM1, an essential enzyme that reversibly catalyzes the conversion of GlcNAc-6-P into GlcNAc-1-P, is found only in eukaryotes because of the above described differences in the synthetic routes of UDP-GlcNAc. AGM1 requires Mg2+ ions to achieve its maximum activity, but the reaction is inhibited by Zn2+ ions (9, 10). This enzyme is a member of the α-D-phosphohexomutase superfamily, which catalyzes an intramolecular phosphoryl transfer on sugar substrates. Although several structures of enzymes in the superfamily have been reported (11–13), the structure of AGM1 has not been determined yet. It is essential to reveal the three-dimensional structure of this enzyme in order to understand its catalytic mechanism and other important differences among enzymes in this superfamily.

Candida albicans is an opportunistic pathogen that causes life-threatening systemic infection in immunocompromised hosts (so called “candidiasis”) (14). It is impossible for fungi to live without biosynthesis of the cell wall, and the chitin of the...
cell wall is synthesized from UDP-GlcNAc. A specific inhibitor of AGM1 from pathogenetic fungi could be a new candidate for an antifungal reagent that inhibits cell wall synthesis. Such a reagent would be expected to have fewer side effects because it acts on the cell wall, which does not exist in humans. In this article, we report the crystal structures of AGM1 from C. albicans (CaAGM1; 544 residues, 60 kDa) for the first time, both in the apo-form and in complexes with substrates (Zn$^{2+}$/PO$_4^{3-}$/GlcNAc-6-P) and products (Zn$^{2+}$/PO$_4^{3-}$/GlcNAc-1-P), and discuss the catalytic mechanism. Although the reaction catalyzed by AGM1 is reversible, in this study we assigned GlcNAc-6-P and GlcNAc-1-P as the substrate and product, respectively, to avoid confusion.

**EXPERIMENTAL PROCEDURES**

**Purification, Crystallization, and Data Collection**—CaAGM1 was overproduced, purified, and crystallized as described previously (6, 15). CaAGM1 was expressed as a glutathione S-transferase fusion and purified with a glutathione-Sepharose 4 FastFlow column (Amersham Biosciences) and a DEAE-Toyopearl 650 column (Tosoh, Tokyo). Crystals were obtained within a week using a reservoir solution (pH 4.6) containing 200 mM NH$_4$H$_2$PO$_4$ and 14–20% (w/v) polyethylene glycol.
Two different types of cryoprotectant solution were prepared. Cryoprotectant 1 contained the reservoir solution and 15% (v/v) glycerol, and cryoprotectant 2, which was used for the preparation of Hg derivatives to avoid precipitation, contained 200 mM acetate buffer (pH 4.5), 20% (w/v) polyethylene glycol 3,350, and 15% (v/v) glycerol. Pt derivatives were prepared by soaking in cryoprotectant 1 with 10 mM K₂PtCl₄ for 7 min. Hg derivatives were prepared by soaking in cryoprotectant 2 with 50% saturated p-chloromercury benzoate for 1 day. The substrate and product complexes were prepared by soaking in cryoprotectant 1 with each ligand (25 mM) and 5 mM ZnCl₂ for 1 min. All crystals were cryocooled in an N₂ gas stream at 95 K. Diffraction data were collected using synchrotron radiation at SPring-8 (BL44B2 with a Mar CCD detector) and at the Photon Factory (BL5 with an ADSC Quantum 315 CCD detector and BL6A with an ADSC Quantum 4R CCD detector) and also using Cu-Kα radiation from a rotating anode generator with RAXIS-IV and RAXIS-VII imaging plate detectors. All data sets were processed and scaled using the programs DENZO and SCALEPACK from the HKL2000 package (16).

Phasing, Model Building, and Refinement — The crystal structure of the apoenzyme was solved by the multiple isomorphous replacement method using the programs SOLVE (17) and RESOLVE (18). The structural model was constructed using the program O (19). The crystal structures of the substrate and product complexes were determined by the molecular replacement method using the apoenzyme structure as a search model by the program MOLREP (20) in CCP4i (21, 22). All structures were refined using the program CNS (crystallography NMR software) (23). The atomic coordinates of CaAGM1 have been deposited in the Protein Data Bank under accession numbers 2DKA (apoenzyme), 2DKC (substrate complex), and 2DKD (product complex). The accessible surface area was calculated using the program SURFACE (24, 25) in CCP4 (21). Structures were superimposed using the program LSQMAN (26). Figures of structures were prepared with the program MOLSCRIPT (27) and Raster3D (28).

RESULTS AND DISCUSSION

Overall Structure of AGM1 — The crystal structure of CaAGM1 was determined at 1.93 Å resolution. Final statistics for all data sets are shown in Table 1. In all structures, approximately 90% of the residues are in the most favored region of the Ramachandran plot defined by PROCHECK (22, 29). It is suggested that the two molecules in the asymmetric unit of the crystal, which are related by noncrystallographic 2-fold symmetry, do not form a biological dimer, because each molecule of this pair buries only 4% of its total solvent-accessible surface in intermolecular contacts, an area outside the range expected for oligomeric proteins (30). Gel-filtration chromatography analysis (data not shown) also supported this hypothesis.

AGM1 contains domains 1 (residues 1–191), 2 (residues 192–311), 3 (residues 312–456), and 4 (residues 457–544). They are arranged in a “heart shape” (Fig. 2, A and B). Domains

![Overall structures of CaAGM1. A and B, front view (A) and top view (B) of the ribbon diagram. Domains 1–4 are colored in green, yellow, red, and blue, respectively. A GlcNAc-1-P molecule and a PO₄⁻ ion are shown as ball-and-stick models, where carbon, oxygen, nitrogen, and phosphorus atoms are presented in black, red, blue, and magenta, respectively. A Zn²⁺ ion is shown as a purple sphere. C, topology diagram of CaAGM1 with the same coloring scheme as in A. Helices are indicated by rectangles and β-strands by arrows. The active-site regions are marked with asterisks. In domains 1–3, the four common strands are numbered, and helices and strands that are not part of the common structure are shown in lighter colors.](image-url)
Comparison with Phosphomannomutase/Phosphoglucomutase (PMM/PGM)—PMM/PGM is also a member of the α-β-phosphohexomutase superfamily and catalyzes the reversible conversion of mannose 6-phosphate into mannose 1-phosphate and glucose 6-phosphate into glucose 1-phosphate (12, 13). PMM/PGM from *Pseudomonas aeruginosa* (PaPMM/PGM) has 463 residues and shares 20.7% sequence identity with CaAGM1 as calculated by the program FASTA (31, 32).

FIGURE 3. Structure-based amino acid sequence alignment between CaAGM1 and PaPMM/PGM. Their secondary structures are shown above and below the sequence alignment, respectively. Helices are indicated by rectangles and β-strands by arrows. Identical residues are indicated by white letters on a red background, and similar residues are indicated by red letters. Active-site loops are outlined in black. PaPMM/PGM shares 20.7% sequence identity with CaAGM1.

1–3 fold similarly, with four β-strands located between two α-helices (Fig. 2C). Domain 4 has a completely different fold, which contains two antiparallel β-sheets. It has less interaction with the other domains and thus is more mobile. In the electron density map of the apoenzyme, domain 4 in one of two noncrystallographic symmetric molecules is disordered. However, in the structures of the substrate and product complexes, the electron density of domain 4 is clearly observed because of the binding of the substrate.
The superposition of CaAGM1 (the product complex) with PaPMM/PGM (the complex with mannose 1-phosphate; Protein Data Bank code 1PCJ) is shown in Fig. 4A. These two structures, which are essentially identical, are composed of four domains arranged in a heart shape. In particular, the active-site residues are well superimposed (Fig. 4B). For AGM1, domain 4 has two additional β-strands (the red region in Fig. 4A), which were not observed in the structures of the superfamily. These strands are superimposed with an α-helix in PMM/PGM.

Circular Permutation—For AGM1 and PMM/PGM, domains 1–3 are structurally similar to each other, each having four β-strands located between two α-helices. The topology
diagrams for domains 2 and 3 are essentially the same between these proteins. The β-strands are arranged in the order of 2–1-3–4, and strand 4 is antiparallel to the other three strands. However, the diagram for domain 1 is completely different, as shown in Fig. 4C. In this domain, the strands are arranged in different order. For AGM1, the β-strands are arranged in the order of 4–3–1–2, and strand 2 is antiparallel to the other three strands. For all of the other proteins in the superfamily, the order is the same as in PMM/PGM (11). The active-site residues are well superimposed, and the sequences around the active-site residues are conserved between AGM1 and PMM/PGM (Fig. 3). It is known that circular permutation can occur by the spatial adjacency of the N and C termini of the polypeptide chain (33, 34). This mutation involves the fusion of the N and C termini and cleavage at another site. Such a mutation is observed in domain 1 of this enzyme (Fig. 4C). The distance on the amino acid sequence between the active-site residues of domains 1 and 2 is 224 residues in AGM1, whereas it is only 134 residues in PMM/PGM. This large gap suggests that AGM1 evolutionarily diverged from the superfamily in the earliest period (35). Although it has been suggested previously that an insert sequence probably exists (36), our structural analysis demonstrates that the difference lies in the strand order.

Active Site—The crystal structures of the complexes with substrates (Zn²⁺/PO₄³⁻/Glcnac-6-P) and products (Zn²⁺/PO₄³⁻/Glcnac-1-P) were also determined at 2.20 and 2.10 Å resolution, respectively (Fig. 5A). The PO₄³⁻ ion was found in these complexes but not in the apoform. The substrate (Glcnac-6-P) and product (Glcnac-1-P) molecules were observed in the active site. The position of the active site in this enzyme is similar to those found in other enzymes belonging to the α-D-phosphohexomutase superfamily (35, 36). The catalytic cleft is formed by four loops from each domain (Fig. 6): the active serine loop (Thr-64–Glu-71 in domain 1), the metal-binding loop (Asp-290–Arg-295 in domain 2), the sugar-binding loop (Glu-387–His-391 in domain 3), and the phosphate-binding loop (Arg-512–Ala-519 in domain 4). The residues identified as affecting the activity by previous mutation analysis are located on all of these loops except the sugar-binding loop (6).

The active serine loop contains the catalytic residue Ser-66 (Fig. 7, A and B). This residue is observed as the phosphorylated form in the superfamily (11, 12). However, judging from the electron density maps, it is not phosphorylated in all structures of the apoform and the substrate and product complexes. In the substrate and product complexes, the Oy atom of Ser-66 is not covalently bonded to the PO₄³⁻ ion. In the apoenzyme, Ser-66 is disordered in one of two noncrystallographic symmetric molecules. On the other hand, in the substrate and product complexes, it is clearly observed but is in the disallowed region of the Ramachandran plot defined by PROCHECK (22, 29) under the influence of the Zn²⁺ ion. The active serine loop is fixed by the Zn²⁺ ion through Ser-66.

The metal-binding loop plays an important role (Fig. 7, A and B). A metal ion is essential for the activity of AGM1. In the apoenzyme, no metal ion is observed at this position, and the side chains of three aspartic acid residues (Asp-290, -292, and -294) on this loop point to various positions. On the other hand, in the substrate and product complexes, the Zn²⁺ ion is clearly observed but is in the disallowed region of the Ramachandran plot defined by PROCHECK (22, 29) under the influence of the Zn²⁺ ion. The active serine loop is fixed by the Zn²⁺ ion through Ser-66.

The metal-binding loop binds to the hydroxyl groups of the substrate (Fig. 7, C and D). In the substrate and product complexes, the side chain of Glu-387 is hydrogen-bonded to the O-3 and O-4 atoms of the sugar ring. In addition, one water molecule is hydrogen-bonded to the O-4 atom in the substrate complex, whereas the side chain of Asn-389 is hydrogen-bonded to the O-3 atom in the
product complex. Compared with the substrate and product complexes, the sugar rings of the substrate (GlcNAc-6-P) and product (GlcNAc-1-P) molecules are related by a 180° rotation around an axis linking the C-4 atom and the midpoint of the C-5—O-5 bond (Fig. 5B). It is suggested that the substrate (the reaction intermediate) rotates 180° around the axis in the reaction.

The phosphate-binding loop, located at the opposite side of the active serine loop, interacts with the phosphoryl group of the substrate (Fig. 7, E and F). This loop is particularly flexible, because in the apoenzyme this loop is not visible in the electron density map. In the substrate and product complexes, however, it is clearly observed (the red dot circle in Fig. 5A). It also plays the role of covering the active-site cleft. The phosphoryl group of the substrate is hydrogen-bonded to the side chains of Arg-512, Ser-514, Gly-515, Thr-516, and Arg-521. The binding modes in the substrate and product complexes are very similar to each other. In addition, two water molecules are hydrogen-bonded to the phosphoryl group in the substrate complex. But only one water molecule binds to the phosphoryl group in the product complex. The N-acetyl group of the substrate in the product complex is replaced by the water molecule in the substrate complex.

**Substrate Specificity**—The N-acetyl group of the substrate is responsible for the substrate specificity (Fig. 7, G and H). In the substrate complex, the carbonyl oxygen atom of the N-acetyl group is hydrogen-bonded to the main chain N atom of Lys-371.
through a water molecule. In addition, the N-acetyl group is located near the side chain of Val-370 at a distance of 3.24 Å. A sufficiently large space is indispensable for the recognition of the substrate. If Val-370 were much larger, steric hindrance would occur. Val-370 of CaAGM1 is replaced by the histidine residue of PMM/PGM, the aspartic acid residue of phosphoglucoaminase mutase, and the tryptophan residue of PGM. Phosphoglucoaminase mutase and PGM are also members of the α-β-phosphohexomutase superfamily. It is difficult for such large residues of other proteins in the superfamily to accept the N-acetyl group. Consequently, it is suggested that a small side chain as the valine residue is suitable to recognize the N-acetyl group of the substrate.

On the other hand, in the product complex, the carbonyl oxygen atom of the N-acetyl group is hydrogen-bonded to a water molecule and the side chain of Asn-389. Interestingly, Asn-389 of CaAGM1 is replaced by the histidine residue of PMM/PGM. Therefore, the carbonyl oxygen atom by itself is too basic to be substituted for the metal ion. Furthermore, as mentioned above, the positive charge from the metal ion could contribute to the transfer of the PO₄³⁻ ion (38, 39).

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