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The Three-dimensional Structure of the N-Acetylglucosamine-6-phosphate Deacetylase, NagA, from Bacillus subtilis

A MEMBER OF THE UREASE SUPERFAMILY

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The enzyme N-acetylglucosamine-6-phosphate deacetylase, NagA, catalyzes the hydrolysis of the N-acetyl group of GlcNac-6-P to yield glucosamine 6-phosphate and acetate, the first committed step in the biosynthetic pathway to amino-sugar-nucleotides. It is classified into carbohydrate esterase family CE-9 (see afmb.cnrs-mrs.fr/CAZY). Here we report the cloning, expression, and three-dimensional structure (Protein Data Bank code 1un7) determination by x-ray crystallography of the Bacillus subtilis NagA at a resolution of 2.0 Å. The structure presents two domains, a (βα)6 barrel enclosing the active center and a small β barrel domain. The structure is dimeric, and the substrate phosphate coordination at the active center is provided by an Arg/His pair contributed from the second molecule of the dimer. Both the overall structure and the active center bear a striking similarity to the urease superfamily with two metals involved in substrate binding and catalysis. PIXE (Proton-Induced x-ray Emission) data show that iron is the predominant metal in the purified protein. We propose a catalytic mechanism involving proton donation to the leaving group by aspartate, nucleophilic attack by an Fe-bridged hydroxide, and stabilization of the carbonyl oxygen by one of the two Fe atoms of the pair. We believe that this is the first sugar deacetylase to utilize this fold and catalytic mechanism.

The first committed step in the biosynthetic pathway to the amino-sugar precursors required for cell wall peptidoglycan and teichioic acid biosynthesis in Bacillus subtilis is the deacetylation of GlcNAc-6-P to yield acetate and GlcN-6-P (Fig. 1). This reaction is catalyzed by the enzyme N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25) NagA (Fig. 16). In Escherichia coli, where the deacetylation of N-acetylglucosamine is additionally important in lipopolysaccharide synthesis and cell wall recycling (4). Neither E. coli nor B. subtilis can convert GlcNac-6-P to GlcNac-1-P directly, so the conversion to GlcN-6-P is a prerequisite for conversion to GlcN-1-P by phosphoglucomutase, which can then be acetylated and uridylated to UDP-N-acetyl-D-glucosamine. Given its position at the crossroads of these bacteria-specific processes, NagA has warranted attention as a potential drug target. Indeed, sugar deacetylation is a validated therapeutic target in other contexts (5, 6).

The enzymes involved in the deesterification/de-N-acetylation of carbohydrates have been classified into 13 families based upon amino acid sequence similarities. The vast diversity of esterases and their frequent lack of specificity, this classification is arguably not as powerful as the related classifications of glycoside hydrolases and glycosyltransferases. However, the enzymes involved in the de-N-acetylation of GlcNac-6-P do all lie in a single sequence-related family termed CE-9. As of September 11, 2003, there are 134 members, the vast majority from bacterial sources. Sequence searches using PSI-BLAST (7) pointed to a three-dimensional structure involving a urease-like bimetallic center different to the known carbohydrate esterases. The majority of carbohydrate esterases/deacetylases whose structures are reported display a classical βα/β “serine protease” fold as revealed by three-dimensional structures of the enzymes from families CE-1 (8), CE-5 (9), CE-7 (10), the plethora of enzymes from family CE-10, and the mycolyltransferase “antigen 85C” (5). A small deviation from this canonical fold is displayed in the CE-12 rhamnogalacturonan acetylemesterase (11). Thus far, the only two “outliers” to this trend are the CE-8 pectin methyl-esterase, which instead presents a twin-aspartate catalytic center grafted upon a right-handed parallel β-helix (12), and LpxC zinc-dependent UDP-3-O-acetyl-N-acetylglucosamine deacetylases from family CE-11, which present an unusual twin-zinc site on a novel αβ framework (6, 13). Here we present the expression and purification of the B. subtilis NagA and an analysis of its metal-ion content through proton-induced x-ray emission data. The three-dimensional structure is reported at 2.0 Å in complex with the reaction product GlcN-6-P (and partial occupancy of unhydrolyzed substrate GlcNac-6-P). The structure reveals NagA to be a member of the “urease superfamily” with a catalytic center involving a binuclear Fe center. The complexes permit a proposal for the catalytic mechanism for NagA equally applicable to other urease superfamily members.

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¶ The abbreviations used are: GlcNAc, N-acetyl glucosamine; GlcN, glucosamine; CE, carbohydrate esterase (deacetylase); PEG, polyethylene glycol; PIXE, Proton-Induced x-ray Emission; ORF, open reading frame; r.m.s., root mean square.

See afmb.cnrs-mrs.fr/CAZY/.
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**EXPERIMENTAL PROCEDURES**

**Cloning and Protein Production—**The nagA gene was cloned into the Gateway Entry vector pDONR201 (Invitrogen) by incorporating attB sequences into the PCR oligonucleotides used to amplify the coding region from chromosomal DNA of *B. subtilis* strain IG20 (168 Trp·-). The cloned fragment was transferred into the T7 promoter-based expression vector pDEST14 by recombination and expressed in *E. coli* BL21(DE3), a strain with an inducible T7 RNA polymerase gene (Novagen).

For protein production, cell cultures were grown in LB medium containing 100 µg/ml ampicillin to an optical density of 0.7 at 600 nm before induction of protein expression by addition of isopropyl-1-thio-
β-D-galactopyranoside to a final concentration of 1 mM. After 3 h of incubation, the cells were harvested by centrifugation and disrupted by sonication. The lysate was clarified by centrifugation, applied to a sonication membrane (Vivaspin), and resolved using a linear gradient of increasing sodium chloride. Fractions containing NagA were concentrated and further purified by gel filtration (Superdex200 HR10/30, Amersham Biosciences) in a 50 mM Tris-HCl (pH 8), 200 mM NaCl buffer with the apparent molecular mass estimated by comparison to standard protein markers. Pure fractions of NagA were pooled, washed into 50 mM Tris-HCl (pH 8) and concentrated to 50 mg/ml using a 10-kDa centrifugation membrane (Vivaspin).

**Peptidoglycan Biosynthesis**

**Teichoic acid Biosynthesis**

**Data quality**

| Resolution of data (Å) | 22–2.00 |
|------------------------|---------|
| Unique reflections | 69,518 |
| Rmerge (outer shell) | 0.109 (0.460) |
| Mean I/σI (outer shell) | 7.5 (2.4) |
| Completeness (outer shell) (%) | 97.4 (94.3) |
| Multiplicity (outer shell) | 2.7 (2.4) |

**Refinement**

**Protein atoms**

5902

**Solvent waters**

626

**Ions**

2Fe

**Rmerge**

0.20

**Rfree**

0.25

**R.m.s. deviation 1–2 bonds (Å)**

0.016

**R.m.s. deviation 1–3 angles (°)**

1.636

**Mean Protein B (Å²) Amol/Bmol**

29.51

**Mean GlcN B (Å²) Amol/Bmol**

26.28

**Mean Solvent B (Å²)**

38

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**Crystal, data, and refinement statistics**

| Crystal parameters |
|--------------------|
| Space group | P2₁,2₁,2 |
| Cell dimensions (Å) | a: 51.68, b: 107.73, c: 188.25 |
| Mean Protein B (Å²) | 2 |
| No. of molecules/AU | 2 |

**TABLE I**

| Parameter | Value |
|-----------|-------|
| λ | 0.9971 |
| Wavelength (Å) | 0.9971 |
| Resolution of data (Å) | 22–2.00 |
| Unique reflections | 69,518 |
| Rmerge (outer shell) | 0.109 (0.460) |
| Mean I/σI (outer shell) | 7.5 (2.4) |
| Completeness (outer shell) (%) | 97.4 (94.3) |
| Multiplicity (outer shell) | 2.7 (2.4) |

**Phasing, Model Building, and Refinement—**The structure was solved by molecular replacement using AMoRe (16) using the structure of the protein from the *Thermotoga maritima* ORF TM0184 (Protein Data Bank code 1012 derived from a Structural Genomics program, website: jcsbg.org/), which has 33% identity with *B. subtilis* NagA, as a search model. The data exhibit pseudosymmetry characterized by a peak at 188.25 Å, corresponding to a solvent content of 60% with two molecules (hereafter designated Amol and Bmol) in the asymmetric unit. All further crystallographic computations were carried out using the CCP4 suite of programs (15).

**Fig. 1. The deacetylation of N-acetylglucosamine-6-phosphate.** a, pathways of amino-sugar metabolism in *B. subtilis*. GlcN and GlcNAc are imported by specific phosphotransferase system components (EC 2.7.1.69) and are converted to precursors of peptidoglycan and anionic polymers essential for cell wall biosynthesis. Proteins catalyzing each step are given with corresponding EC numbers. There seems to be at least three *B. subtilis* gene products able to acetylate GlcN-1-P (PksF, AcuA, and BkdB). Abbreviations: Fru, fructose; Glc, glucose. Gene products: NagB, GlcN-6-P isomerase; GlmS, L-glutamate-δ-fructose-6-P amidotransferase; GlmM, phosphoglucomutase; GcaD, UDP-GlcNAc pyrophosphorylase; Pgi, Glc-6-P isomerase; GtaB, UTP-Glc-1-P uridylyltransferase. b, the chemical reaction catalyzed by NagA, the N-acetylglucosamine-6-phosphate de-N-acetylase.
The electron density map calculated from the model was difficult to interpret. Several cycles of the REFMAC/ARP-WARP program in warpNtrace mode (17, 18) resulted in the automatic building of the main chain of most of the α-helices and β-sheets, but attempts at automatic assignment of the sequence failed. Manual rebuilding was therefore performed with the X-Autofit module in the program Quanta (Accelrys Inc., San Diego, CA). The model was refined using REFMAC (17) interspersed with manual rebuilding using X-Autofit in Quanta. The final model contains 5902 non-hydrogen protein atoms, 626 water molecules, 4 metal-ions, 2 polyethylene glycol molecules, and 2 GlcN-6-P molecules. The crystallographic R-factor and R-free values (19) are 0.20 and 0.25, respectively (Table I). The stereochemistry of the model was assessed with the program PROCHECK (20) prior to deposition.

**RESULTS**

**Overall Structure**—The *B. subtilis* NagA exists as a dimer in solution as determined by gel filtration and dynamic light scattering data (data not shown). The crystals of NagA contain two molecules in the asymmetric unit. The two molecules involved in what we believe is the active dimer (see below) are related by the crystallographic 2-fold symmetry around the c axis. The polypeptide chain is visible from residues 3 to 394 in each molecule. The monomer folds into two structural domains (Fig. 2a), an (α/β)$_8$ barrel (residues 59–332) enclosing the catalytic site of the enzyme and a small β-strand barrel made up from secondary structure elements contributed by the N and C termini (residues 3–58 and 368–394). The secondary structure of NagA as defined by the program DSSP (23) is shown in Fig. 2b. The (α/β)$_8$ barrel is a somewhat distorted “TIM-
**NagA Deacetylase**

**Fig. 3.** Electron density at the active center of B. subtilis NagA, *a*, the two Fe sites with their coordinating residues. *b*, the reaction product GlcN-6-P. Arg-324 and His-233 shown in purple are derived from the second molecule of the dimer. This figure in divergent (“wall-eyed”) stereo was drawn using PyMOL (pymol.sourceforge.net) and shows maximum likelihood σA (50) weighted 2Fobs − Fcalc electron density at 0.25 electrons/Å².

barrel” fold, the connections between the β-strands and the helices are equivalent apart from an excursion of a small three-stranded β-sheet between β_4 and α_6 that closes one end of the (α/β)_6 barrel and contribute residues involved in ligand interaction (see below). The second domain is an eight-stranded sheet that wraps into an incomplete barrel. Five of the β-strands come from the N-terminal region of the enzyme (βA to E) and form the main part of the β barrel with two curved β-strands (βA and B). Finally, three other β-strands lie “on top” of the others and belong to the C-terminal region of the enzyme (βK to M). A DALI search (24) reveals a structural relationship between the catalytic domain and a large family of metal-dependent hydrolases with a similar barrel as described by SCOP (25), which includes urease, phosphotriesterase, and hydantoinase (26, 27). A subset of this family, now including NagA, is characterized by the presence of an all-β-strand domain made up of composite elements from the N and C termini (25).

The interface-accessible surface area is 1054 Å², which represents 7% of the total (28). The two molecules are related by 180°-rotational symmetry, and a loop between β_6 and α_6 forms an arm, which contacts its neighbor subunit across the dimer interface. There are two salt bridges between Asp-246 and Lys-265 and a number of hydrogen bond contacts between α_6 and α_7 to their symmetric equivalents with water excluded from the interface.

A comparison with the original search model derived from the *T. maritima* ORF TM0184, which crystallized in space group P3_21, shows a very similar fold with an r.m.s. deviation of 1.96 Å for 354 C atoms of the monomer (calculated with the program QUANTA (29)). The relative orientation of the two molecules of the *T. maritima* dimer is slightly different and reflected in an r.m.s. deviation of 6.18 Å for 706 C atoms of the dimer. The highest deviations occur in the β barrel domain (−6.36 Å for βC, βD, and βE) and in the loops connecting α_2 to β_3 and α_4 to β_5 (−5 Å). Only one iron ion is observed in the structure of ORF TM0184, and it superposes with Fe-2 of NagA and interacts with the same residue types (His-176, His-197, and Glu-115). The implications of this finding are described below.

**The Catalytic Site and Substrate Binding**—Electron density, corresponding to the reaction product GlcN-6-P, was clearly visible in the cavity of the (α/β) barrel (Fig. 3b) (30). In one molecule, there is residual difference density suggesting a partial (<0.3) occupancy of unhydrolyzed substrate, GlcNAc-6-P, and although partial occupancy of substrate sheds light on catalysis (see “Discussion”), it was not refined due to the constraint of limited resolution. GlcN-6-P binds in the cavity through side chain interactions with Asn-226 to the phosphate group, His-258 to the 1'-sugar hydroxyl, and main chain hydrophobic bonds (Fig. 4). A putative catalytic acid (discussed below), Asp-281, is positioned close to the scissile bond. Strikingly, the phosphate also makes contact with a pair of side chains, His-233 and Arg-234, which form a pincer-like extension from the βC-α_6 loop contributed from the partner subunit (Fig. 5). In the *T. maritima* structure, the equivalent residues (His-207 and Arg-208) point toward the solvent, suggesting that they are solicited only in the presence of the ligand in the cavity. The recruitment of residues from an adjacent partner subunit in a homo-oligomer toward substrate binding is unusual but not unique. The 180°-rotational symmetry between two monomers of granzymeA is used in the functional dimer to form an extended substrate-binding cleft (31, 32). Examples of an Arg residue cooperating (from adjacent subunits in a hexamer) to ligate a substrate phosphate ion include uridine phosphorylase (33) and purine nucleoside phosphorylase (34).

Additional electron density at the active center was assumed to correspond to bound metal-ions. The microPIXE technique applied to elemental analysis of proteins (35) was used to determine the metal content. To estimate metal content, a
native crystal grown from lithium acetate was used, which revealed the presence of phosphorus, sulfur, chlorine, potassium, calcium, manganese, iron, copper, and zinc. Because this sample had no additional sulfur in the buffer, the number of atoms for these elements per protein molecule (i.e. per 12 sulfur atoms) could be calculated and averaged from individual point spectra: iron 1.3 (±0.2); zinc 0.21 (±0.03); manganese 0.17 (±0.02); potassium 0.11 (±0.02); and copper 0.05 (±0.02). Because iron was the predominant metal atom found in the purified protein (grown from E. coli in LB medium), iron was modeled in the electron density. The two Fe ions are situated in the bottom of the active site. One is more buried (Fe-2) and makes four interactions with protein side chains (His-63 and His-65 on β3, Glu 136 from the end of strand 3 and the active site Asp-281), and the other (Fe-1) is held in position by His-223, His-202, and Glu-136. The two metal atoms are 3.5 Å apart and refine with B-factors of 26 Å² for Fe-2 and 21 Å² for Fe-1 in each molecule of the asymmetric unit.

A comparison of NagA protein sequences from Gram-negative, Gram-positive, and archeal bacteria highlights other residues important for NagA structure and function (Fig. 6). There are four residues in the NagA structure whose backbone dihedral angles fall outside of the normal range as defined by a Ramachandran plot (36). A cis-proline (Pro-138) is conserved and occurs in a Gly-Pro motif immediately after Glu-136 at the end of β3, which bridges the two Fe ions. The metal-binding His-223 is held at an unusual angle (ϕ = 26.1, ψ = 72.2) by the side chain of Glu-251, and Tyr-225 (ϕ = 86.7, ψ = −27.5) also displays a high energy conformation, which allows the substrate-binding Asn-226 side chain to turn back toward the substrate. Ser-344 (ϕ = −152.1, ψ = −59.3) mediates a tight turn between αA and αB. The substrate-binding residue His-258 is held in position by the side chain of Asp-255, which is conserved. The barrel is completed by an interaction between the conserved residues Thr-280 (β6) and Asp-61 on strand β1. The alignment also highlights some potential differences between NagA sequences of B. subtilis and E. coli. The inter-subunit salt linkage, which is conserved between B. subtilis and T. maritima NagA proteins, appears to be absent in the same position of the E. coli sequence. Although the phosphate-binding residue Arg-234 is conserved in all of the three sequences, its partner, His-233, is not present in E. coli NagA. Perhaps the biggest difference is that Gln and Asn residues in E. coli NagA replace the residues His-63 and His-65, which bind Fe-2 and coordinate the active site Asp-281.

**DISCUSSION**

The structural superfamily of metal-dependent hydrolases includes enzymes with highly diverse substrates. This family has been coined an “evolutionary treasure” (26). The trove has expanded with structural characterization of predicted family members (27) including isoaaspartylpeptidase (37), N-acyl-d-aminoacylase (38), renal dipeptidase (39), and now NagA. A recurrent feature in the family is the post-translational modification of a lysine residue on strand β2 to a carboxylated form, which serves to bridge two metal-ions in the catalytic site. In an interesting variation, both NagA and renal dipeptidases have exploited glutamate carboxylates in a similar spatial position,
which are instead donated from strand $\beta_3$ of the $\beta$ barrel to serve the same role, which may be one reason that NagA was not predicted as a family member by the original grouping based on primary sequence (26).

Another unusual feature of NagA is that across species there are inconsistencies in the gene order and chromosomal operon structure. In *E. coli*, nagA is in an operon with components of the import GlcNAc phosphoenolpyruvate-phosphotransferase system. Although the same is true in some Gram-positive organisms, such as *Bacillus sphaericus* (40), many Bacillus nagA genes are in a short operon with nagB, which encodes a GlcN-6P deaminase/epimerase. In *B. subtilis*, the two genes overlap by 2 bp and in an extreme case by 13 bp in *Bacillus halodurans*, strongly suggesting that they are co-transcribed and translated. Indeed, there may be some real differences between the GlcNAc deacetylase/GlcN epimerase systems.
across the bacterial order divides. The archaeabacteria *T. maritima* lacks a phosphotransferase system, and its *nagA* gene is not genetically linked to *nagB*. The Gram-negative *NagA* proteins, which have been characterized, appear to be functional as homotrimers (1, 2). The *E. coli* GlcN-6P epimerase is a hexamer that displays allosteric activation (41) and has greater sequence identity to *NagB* from eukaryotic sources than to the *B. subtilis* *NagB*, which is a monomer.3 It is clear that the role and regulation of the *NagA/NagB* systems is a complex area that demands further investigation.

A Putative Reaction Mechanism for *NagA*—There is little consensus in the literature concerning the reaction mechanism of urease superfamily members. Even for urease itself, a number of different mechanisms have been proposed (42–45), none of which is compatible with the active center of *NagA*. What is clear is that the active site must provide at least two functions: (a) a catalytic (Brønsted) acid to protonate the leaving group (*pKₐ > 30*) to facilitate its departure and (b) a water molecule, which must in some way be activated through deprotonation, or hydroxide-ion to perform the hydrolytic attack at the carbonyl carbon of the substrate. For these superfamily members acting on acetyl or *N*-acetyl substrates, it is likely that some means of stabilizing the developing negative charge on the carbonyl oxygen must be provided in a manner analogous to the “oxynion hole” of serine proteases. There does seem general consensus as to the latter function. Ni-1 of ureases analogous to Fe-1 in *NagA* (44, 45) seems perfectly positioned to stabilize the carbonyl group of *NagA*. Ni-1 of ureases analogous to Fe-1 in *NagA* as this space is instead occupied by an amine leaving group via Asp-281 and stabilization of the tetrahedral intermediate through coordination of Fe-1 with the polarized carbonyl oxygen. We hope that further studies with inhibitors and putative transition-state mimics will lead to a greater understanding of catalysis in this unusual enzyme.

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