doi:10.1016/j.jmb.2006.03.024 J. Mol. Biol. (2006) 359, 308–321

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Structural Analysis of *N*-acetylglucosamine-6-phosphate Deacetylase Apoenzyme from *Escherichia coli*

Frederico M. Ferreira[1], Guillermo Mendoza-Hernandez[2] Maria Castañeda-Bueno[2], Ricardo Aparicio[3], Hannes Fischer[1] Mario L. Calcagno[2] and Glaucius Oliva[1]*

[1]*Instituto de Física de São Carlos, Universidade de São Paulo, C.P. 369, 13560-970 São Carlos, SP, Brazil*

[2]*Departamento de Bioquímica Facultad de Medicina Universidad Nacional Autónoma de Mexico AP. Postal 70-159 Cd. Universitaria, 04510 Mexico, DF Mexico*

[3]*Instituto de Química Universidade Estadual de Campinas, C.P. 6154, 13084-862 Campinas, SP, Brazil*

**Corresponding author*

We report the crystal structure of the apoenzyme of *N*-acetylglucosamine-6-phosphate (GlcNAc6P) deacetylase from *Escherichia coli* (*Ec*NAGPase) and the spectrometric evidence of the presence of Zn^{2+} in the native protein. The GlcNAc6P deacetylase is an enzyme of the amino sugar catabolic pathway that catalyzes the conversion of the GlcNAc6P into glucosamine 6-phosphate (GlcN6P). The crystal structure was phased by the single isomorphous replacement with anomalous scattering (SIRAS) method using low-resolution (2.9 Å) iodine anomalous scattering and it was refined against a native dataset up to 2.0 Å resolution. The structure is similar to two other NAGPases whose structures are known from *Thermotoga maritima* (*Tm*NAGPase) and *Bacillus subtilis* (*Bs*NAGPase); however, it shows a phosphate ion bound at the metal-binding site. Compared to these previous structures, the apoenzyme shows extensive conformational changes in two loops adjacent to the active site. The *E. coli* enzyme is a tetramer and its dimer–dimer interface was analyzed. The tetrameric structure was confirmed in solution by small-angle X-ray scattering data. Although no metal ions were detected in the present structure, experiments of photon-induced X-ray emission (PIXE) spectra and of inductively coupled plasma emission spectroscopy (ICP-AES) with enzyme that was neither exposed to chelating agents nor metal ions during purification, revealed the presence of 1.4 atoms of Zn per polypeptide chain. Enzyme inactivation by metal-sequestering agents and subsequent reactivation by the addition of several divalent cations, demonstrate the role of metal ions in *Ec*NAGPase structure and catalysis.

Keywords: *N*-acetylglucosamine-6-phosphate deacetylase; amino sugar catabolism; amidohydrolases; PIXE; ICP-AES

Introduction

The bacterium *Escherichia coli* displays a great versatility in the utilization of different carbon sources and it can use a great variety of carbohydrates and related compounds as sources of energy. When amino sugars, glucosamine (GlcN) or *N*-acetylglucosamine (GlcNAc), are present in the environment, they are taken up by the cells and used for cell wall and lipid A synthesis as well as a carbon source. Under these conditions, the synthesis of the enzyme glucosamine synthase (GlmS) necessary for the biosynthesis of amino sugars, is repressed while the enzymes necessary for the transport and metabolism of amino sugars are induced.[1,2] GlcNAc is amongst the best carbon sources known for *E. coli*, producing similar growth rates as glucose. In *E. coli* both GlcN and GlcNAc are phosphotransferase

Abbreviations used: GlcNAc6P, *N*-acetylglucosamine 6-phosphate; NAGPase, *N*-acetylglucosamine-6-phosphate deacetylase; SIRAS, single isomorphous replacement with anomalous scattering; SAXS, small-angle X-ray scattering; PIXE, photon-induced X-ray emission; ICP-AES, inductively coupled plasma emission spectroscopy; DRM, dummy residues model.

E-mail address of the corresponding author: oliva@if.sc.usp.br

system (PTS) sugars, so their transport into the cell is coupled to their phosphorylation. The *N*-acetylglucosamine 6-phosphate (GlcNAc6P) resulting from the transport of GlcNAc is hydrolyzed to GlcN6P and acetate by the enzyme GlcNAc6P deacetylase (EC 3.5.1.25) encoded by the gene *nagA*. GlcN6P, either produced from GlcNAc6P or from the transport of GlcN, is then deaminated and isomerized by the enzyme GlcN6P deaminase (EC 3.5.99.6, encoded by *nagB*), which produces free ammonia and fructose 6-phosphate.[3] Utilization of GlcNAc has also been reported for other pathogenic bacteria, such as *Klebsiella pneumoniae* and *Vibrio choleræ* and it was demonstrated that it induces cellular morphogenesis in *Candida albicans* by disruption of the *N*-acetylglucosamine catabolic pathway.[4] In the absence of an exogenous supply of amino sugars, GlcN6P can be synthesized from Fru6P and glutamine by the enzyme glutamine-fructose-6-phosphate aminotransferase (GlmS, E.C. 2.6.1.16), encoded by one of the genes in the biosynthetic operon, *glmUS*. In the presence of an exogenous supply of amino sugars the expression of the biosynthetic operon is repressed while the expression of the genes necessary for GlcNAc utilization as a carbon source are induced. The genes for the transport (*nagE*) and catabolism (*nagBA*) of GlcNAc are organized in two divergent operons *nagE-nagBACD*. The inducing signal turning on expression of the *nagE-nagBA* genes is GlcNAc6P, which prevents the transcriptional repressor NagC from binding to its operators.[1] GlcNAc6P that is the direct product of GlcNAc transport, has an additional regulatory role; it is the allosteric activator of GlcN6P deaminase (NagB).[5,6] *Ec*NAGPase is not allosteric, but it is a key component of the coordinated control of amino sugar metabolism, because of the double regulator role of its substrate. The operation *in vivo* of both the transcriptional and allosteric control of amino sugar utilization in *E. coli* has been recently demonstrated.[7] Although *Ec*NAGPase is primarily a catabolic enzyme, recently it has been shown to have an important role in the amino sugar salvage pathway of peptidoglycan recycling in *E. coli*.[8] At each generation, *E. coli* breaks down about 60% of its cell wall peptidoglycan, most of which is efficiently recycled in a sequence of reactions that release GlcNAc and 1,6-*anhydro*-*N*-acetylmuramic acid in the cytoplasm.[9] The GlcNAc recycling pathway involves the phosphorylation of these intermediates by two specific kinases, NagK and AnmK,[9,10] and its conversion into GlcN6P by NAGPase (NagA), which is subsequently isomerized to GlcN1P (GlmM) and then N-acetylated with the participation of acetylcoenzyme A (GlmU).[11,12] In eukaryotic organisms GlcNAc6P is directly isomerized into GlcNAc1P;[13] this metabolic difference between animals and bacteria points toward bacterial NAGPases as potential drug targets. *Ec*NAGPase has been obtained pure from an overproducing *E. coli* strain and shown to be a tetramer of identical subunits of 41 kDa and 382 residues. Its kinetics was studied both in the forward and the backward directions of the reaction and a kinetic mechanism was proposed.[14] Although the reverse acetylation reaction was demonstrated *in vitro*, there is no evidence that it could have a physiological role *in vivo*. The crystal structures of the NAGPases from *Thermotoga maritima* (PDB code 1O12, 2.5 Å resolution) and *Bacillus subtilis* (PDB code 1UN7, 2.0 Å resolution)[15] have been deposited into the Protein Data Bank (PDB). Simultaneously with the deposition of the present structure (PDB code 1YRR, 2.0 Å resolution), a new entry for the *E. coli* deacetylase also appeared (PDB code 1YMY, 2.6 Å resolution). The *B. subtilis* enzyme contains a binuclear iron center, whereas the *T. maritima* structure has only one iron atom bound. These structures show that the GlcNAc6P deacetylases belong to the structural superfamily of metal-dependent amidohydrolases,[15] whose members can bind one, two or three metal atoms in the active site, with a wide repertoire of possible metals, including Fe^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+}, amongst others.[16] The enzymes from different organisms show variable oligomeric organization. As in *E. coli*, the NAGPases purified from the bacteria *Thermus caldophilus*,[17] *V. choleræ*,[18] and *Alteromonas* sp.[19] are tetrameric proteins, in contrast with *B. subtilis* deacetylase that has been shown to be a dimer.[15] NAGPases from *B. subtilis* and *T. maritima* are iron-proteins and there is indirect evidence that those from *Ateromonas* sp., *V. choleræ* and *T. caldophilus* are metalloproteins, although the involved metal was not identified. Here, we present the crystallographic structure of GlcNAc6P deacetylase from *E. coli* at 2.0 Å resolution as a tetrameric particle and in the metal-free state. However, both experiments, photon-induced X-ray emission (PIXE) and inductively coupled plasma emission spectroscopy (IC-PES), show that the native *Ec*NAGPase contains Zn atoms. Enzyme inactivation by metal-sequestering agents and subsequent reactivation by several divalent cations were used to explore the role of the metal ligands in catalysis by the enzyme.

Results and Discussion

The quality of the model

The crystal structure of *Ec*NAGPase was determined by the single isomorphous replacement with anomalous scattering (SIRAS) method using low-resolution (2.9 Å) anomalous scattering of iodine. The positions of 94.7% of the expected non-hydrogen protein atoms were identified and refined up to 2.0 Å resolution (Table 1A). Model building and refinement improved the R and R_{free} factors from 30.3% and 26.7% to 19.9% and 15.9%, respectively. The model presented very good stereochemical parameters (Table 1B). The r.m.s. deviation for bond distances was 0.022 Å and for bond angles was 1.89°. The average B-factor over all atoms was 21.2 $Å^2$. Most of the amino acid residues have their main-chain Φ/Ψ angles lying in the favored regions of the Ramachandran plot[20] (Table 1C). Only four amino acid residues (Asp70A, Asp70B, Val138A, and Tyr218B) were