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Purification, crystallization and preliminary X-ray analysis of NtdA, a putative pyridoxal phosphate-dependent aminotransferase from *Bacillus subtilis*

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NtdA is a putative sugar aminotransferase that is required for the synthesis of 3,3′-neotrehalosadiamine. The enzyme was purified to homogeneity by means of Ni^{2+}-affinity chromatography and was crystallized using the microbatch method. X-ray diffraction data were collected from a single crystal to 2.3 Å resolution at the Canadian Light Source (CLS). The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 50.3$, $b = 106.7$, $c = 96.7$ Å, $\beta = 96.2^\circ$, and contained two molecules per asymmetric unit.

1. Introduction

3,3′-Neotrehalosadiamine (NTD; Fig. 1) was originally identified as an amino-sugar antibiotic produced by *Bacillus pumilus* (Tsuno *et al.*, 1986) and *B. circulans* (Numata *et al.*, 1986) and was shown to inhibit the growth of *Staphylococcus aureus* and *Klebsiella pneumonia*. The operon involved in the biosynthesis of NTD encompasses the *ntdABC* genes. Genome sequencing revealed that *B. anthracis* and *B. licheniformis* also contain orthologues of the NTD biosynthetic enzymes and the transcriptional regulator NtdR (Inaoka & Ochi, 2007). NTD acts as an autoinducer for its own biosynthesis genes *via* the regulator protein NtdR encoded by *ntdR* (Inaoka *et al.*, 2004). Inaoka and coworkers also showed that the operon, which is dormant in *B. subtilis*, is activated by a mutation in the β-subunit of RNA polymerase, resulting in NTD production. The *ntdABC* operon is repressed by GlcP-dependent glucose uptake (Inaoka & Ochi, 2007). Expression of the *ntdABC* biosynthetic operon in *Escherichia coli* resulted in NTD production, suggesting that the *ntdABC* operon encodes all the enzymes necessary for production of NTD (Inaoka *et al.*, 2004). Sequence comparisons suggested that the gene products of *ntdA*, *ntdB* and *ntdC* are a pyridoxal 5′-phosphate (PLP) dependent aminotransferase, an HAD hydrolase superfamily member and an NADH-dependent dehydrogenase, respectively. However, no further details have been established regarding the reactions or intermediates in the biosynthetic pathway. In order to understand this pathway, a detailed knowledge of the biosynthetic role of each enzyme in the production of NTD is required. High-resolution structures of the enzymes involved in the biosynthesis of NTD would give us a better understanding at a molecular level of the mechanisms and substrate preference of these enzymes.

The NTD-biosynthesis operon gene *ntdA* is the first gene of the *ntdABC* operon. Based on sequence alignment, NtdA has been postulated to be a sugar aminotransferase belonging to group VI of the aminotranferase family (Hwang *et al.*, 2004, 2005) that catalyses

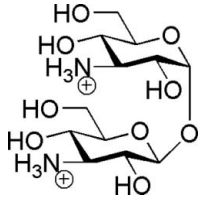
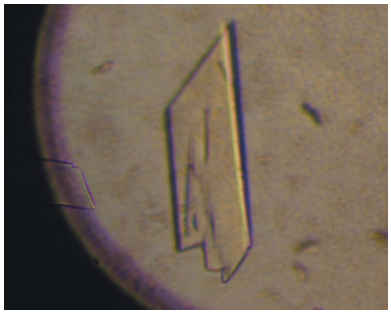


Figure 1
3,3′-Neotrehalosadiamine (NTD). Although the biosynthetic pathway is unknown, the involvement of the PLP-dependent NtdA suggests that the 3-amino groups are introduced into a glucose or neotrehalose derivative containing a 3-keto group or groups.



the PLP-dependent conversion of a 3-keto sugar derivative to the corresponding 3-amino compound. However, at present neither the amino donor nor the sugar substrate is known. A *PSI-BLAST* (Altschul *et al.*, 1997) search using the NtdA sequence as a query suggests that NtdA is a member of the DegT/DnrJ/EryC1/StrS subfamily, a group of sugar aminotransferases (SATs) with 30–58% sequence identity to NtdA. A search with the NtdA sequence against the Protein Data Bank (PDB) identified several SATs with known structure and function: DesI (sharing 29% sequence identity with NtdA; PDB code 2po3) and DesV (33% identity; PDB code 2oga) from *Streptomyces venezuelae*, QdtB (34% identity; PDB code 3frk) from *Thermoanaerobacterium thermosaccharolyticum*, ArnB (31% identity; PDB code 1md0) from *Salmonella typhimurium*, GDP-perosamine synthase from *Caulobacter crescentus* (33% identity; PDB code 3bn1) and BtrR from *B. circulans* (30% identity; PDB code 2c7t). All these enzymes catalyze a PLP-dependent amination of a 3-keto or 4-keto sugar. In each case the amino donor is either glutamate or glutamine and the amino acceptor (*i.e.* the ketone) is a sugar nucleotide, except in the case of NtrR, which uses *scyllo*-inosose (Hwang *et al.*, 2005). Structural information on these SATs revealed that they are functional homodimeric enzymes, *e.g.* residues from both subunits form the active sites in the dimer. SATs have been classified into three subgroups based on multiple sequence alignment: VI_α, the members of which utilize NDP-4-keto sugars, VI_β, utilizing NDP-3-keto sugars, and VI_γ, which utilize *scyllo*-inosose (Hwang *et al.*, 2004, 2005). In terms of nucleotide specificities, all the characterized sugar substrates of the VI_β subgroup are TDP-3-keto sugars (Burgie *et al.*, 2007; Chen *et al.*, 1999; Rodriguez *et al.*, 2006; Weber *et al.*, 1990; Pföstl *et al.*, 2008; Pfoestl *et al.*, 2003; Hwang *et al.*, 2004). NtdA could be classified as a SAT VI_β enzyme as NtdA is proposed to aminate position 3 of a glucose derivative. In order to characterize NtdA and test our hypothesis, our goal is to discover the precise reaction catalyzed by NtdA using purified recombinant enzyme to perform *in vitro* experiments and by solving the crystal structure with and without putative substrates and amino donors.

This paper describes the subcloning and expression of the *ntdA* gene and the purification, crystallization and preliminary X-ray analysis of hexahistidine-tagged NtdA from *B. subtilis*, a putative PLP-dependent aminotransferase that is involved in the biosyntheses of NTD.

2. Material and methods

2.1. Expression and purification

The gene encoding *ntdA* was a gift from Dr Kozo Ochi (National Food Research Insititute, Tsukuba, Japan). In order to subclone the gene into the *Nde*I and *Bam*HI sites of pET-28b (Novagen) for expression as an N-terminal hexahistidine-fusion protein, an internal *Nde*I site was removed by silent mutation (T1152C) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The mutants were screened by *Nde*I digestion. The *ntdA* gene was then PCR-amplified, incorporating *Nde*I and *Bam*HI sites at the 5′- and 3′-ends, respectively. The resulting 1365 bp PCR product was digested with the appropriate restriction enzymes and ligated with pET-28b that had also been treated with *Nde*I and *Bam*HI. Transformation of *Escherichia coli* XL1-Blue (Stratagene) resulted in colonies on LB-agar plates containing kanamycin; colonies were cultured in LB medium containing 50 µg ml^{-1} kanamycin and plasmid DNA extraction allowed screening of transformants by restriction digest. Positive clones were confirmed by DNA sequencing.

E. coli strain BL21-Gold (DE3) (Stratagene) was used to overexpress *ntdA*. Cells containing the *ntdA*-pET28b expression construct for overexpression of N-terminally His_6-tagged NtdA were cultured at 310 K and 225 rev min^{-1} in LB medium containing 50 µg ml^{-1} kanamycin. Expression was induced by the addition of 1 m*M* IPTG at an OD_{600} of ∼0.5. Cells were grown for an additional 5 h until an OD_{600} of 1.6 was reached. The cells were harvested by centrifugation at 4000*g* for 45 min and resuspended in lysis buffer (buffer *A*) containing 5 m*M* imidazole, 0.50 *M* NaCl, 20 m*M* Tris pH 8.0. After the addition of 2 m*M* lysozyme, 1 m*M* AEBSF and catalytic amounts of DNAse, the cells were incubated on ice for 30 min. The cells were then lysed on ice by sonication using a Virsonic 600 (VirTis) at 20% amplitude for 2 min using a cycle of 5 s on and 5 s off. Cell debris was then removed by centrifugation at 4000*g* for 45 min at 277 K. The NtdA-containing supernatant was subjected to a 10% ammonium sulfate precipitation for 30 min at 277 K to remove high-molecular-weight contaminating proteins. The supernatant was clarified by centrifugation at 12 000*g* for 20 min at 277 K. After filtration through a 0.22 µm filter (Amicon), the sample was loaded onto a 10 ml Ni^{2+}-chelating column pre-equilibrated with buffer *A* at 295 K. The column was washed with eight column volumes of buffer *A* to remove unbound proteins. NtdA was eluted with a 20 column-volume linear imidazole gradient from 5 to 800 m*M* imidazole in 20 m*M* Tris pH 8.0 containing 0.5 *M* NaCl at a flow rate of 10 ml min^{-1}. His_6-tagged NtdA eluted at about 150 m*M* imidazole. The purity of the protein-containing fraction was analyzed by SDS–PAGE. Fractions containing pure His_6-NtdA (molecular mass 50.96 kDa) were pooled and dialyzed against 150 m*M* NaCl, 25 m*M* Tris pH 8.5. His_6-tagged NtdA was concentrated on a 30K Amicon centrifuge filter device to a concentration of 8.0 mg ml^{-1}. The amount of protein was determined using UV absorption spectroscopy at 280 nm with a theoretical molecular extinction coefficient of 37 820 M^{-1} cm^{-1}. A 1 l LB culture produced ∼18 mg pure protein.

2.2. Crystallization

Crystals of NtdA were grown at 295 K using the microbatch method (D'Arcy *et al.*, 2004) and 72-well hydrophobic microbatch plates (Hampton Research). Initial screening for crystallization conditions was performed using commercial crystallization screens (Qiagen). Equal volumes of protein solution (1.2 µl) and precipitant solution (1.2 µl) were mixed. The crystallization drop was overlaid with paraffin oil (Hampton Research) to prevent the evaporation of water from the drop. Very small thin plate-like crystals grew from trisodium citrate pH 5.6 using salts and polyethylene glycol (PEG; PEG 3350, PEG 4000 and PEG 8000) as the precipitant. The best-looking crystals were obtained from conditions containing either 15 or 30%(*w*/*v*) PEG 4000, 0.2 *M* ammonium acetate, 100 m*M* trisodium citrate pH 5.6 (from the Classic and Classic Lite crystallization screens; Qiagen). Optimization to improve the crystal quality was performed with the Additive Screen (Qiagen) using the microbatch method. Equal volumes of protein solution (1.2 µl) and additive solution in 100 m*M* trisodium citrate pH 5.6 and 20% PEG 3350 (1.2 µl) were mixed and overlaid with paraffin oil. Well defined single plate-like crystals were obtained with 0.2 *M* NaCl and 0.2 *M* ammonium acetate as additives. The best crystals were finally obtained with 0.2 *M* ammonium acetate and with 0.4 *M* NaCl in 100 m*M* trisodium citrate pH 5.6 and by slightly decreasing the PEG concentration to 17.5%. These plate-like crystals appeared after 1 d and grew to dimensions of about 0.2 × 0.5 × 0.05 mm within 4 d (Fig. 2).

2.3. Data collection and processing

For data collection, a crystal was transferred for a few seconds into mother liquor containing 25% glycerol as a cryoprotectant. The crystal was mounted on a litho-cryoloop (Molecular Dimensions Ltd) and subsequently flash-cooled in liquid nitrogen. X-ray data were collected to 2.3 Å resolution at 100 K in a cold nitrogen stream on beamline 08-ID.1 at the Canadian Light Source (CLS). A total of 360 images were collected using a MAR225 CCD X-ray detector; each was exposed for 2 s with a 1.0° oscillation at a crystal-to-detector distance of 250 mm. The diffraction images were processed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Structure determination

NtdA has been purified to homogeneity by means of Ni^{2+}-affinity chromatography. A UV–vis absorption scan of the purified enzyme showed absorption peaks at 330 and 410 nm which were indicative that the cofactor PLP had copurified (Zhang *et al.*, 2005). The enzyme was crystallized and a single data set was collected to 2.3 Å resolution at the CLS. Autoindexing of the diffraction data using *HKL*-2000 showed that the crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 50.3, b = 106.7, c = 96.7 Å, β = 96.21°. The asymmetric unit appeared to contain one dimer, giving a Matthews coefficient of 2.60 $Å^3$ Da^{-1} and a solvent content of 52.8% (Matthews, 1968). Examination of the self-rotation function in $P2_1$ clearly showed noncrystallographic twofold symmetry. The preliminary X-ray crystallographic statistics are summarized in Table 1.

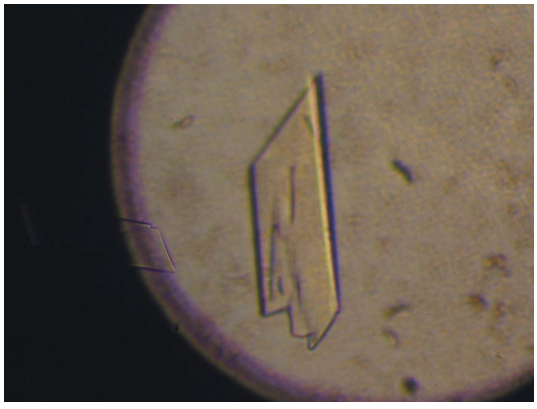


Figure 2
Crystals of NtdA. The crystals have approximate dimensions of 0.2 × 0.5 × 0.05 mm.

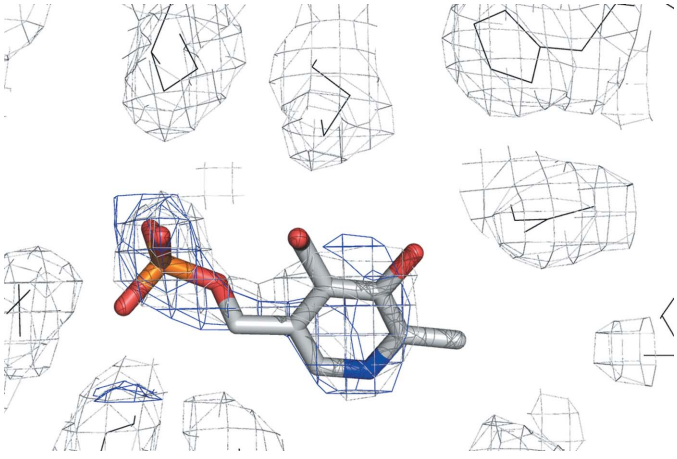


Figure 3
Initial density maps to 2.3 Å resolution generated from the molecular-replacement solution from *MrBUMP*. The map in light grey is the $2F_o - F_c$ map contoured at 1σ. The map in blue is the $F_o - F_c$ map contoured at 3σ. The actual model is shown as black lines, while PLP (not included in the initial model, but inserted to show location) is shown as a stick model. The figure was generated using *PyMOL* (DeLano, 2002).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	08ID-1, CLS
Wavelength (Å)	0.98066
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 50.3, b = 106.7, c = 96.7, β = 96.2
Resolution (Å)	30.0–2.3 (2.38–2.30)
Observed reflections	188518 (12736)
Unique reflections	45037 (3746)
Completeness (%)	98.0 (82.3)
Multiplicity	4.2 (3.4)
$I/\sigma(I)$	15.9 (3.5)
R_{merge} (%)	7.8 (27.2)
Mosaicity	0.49

3.1. Molecular replacement

Molecular replacement used the program *MrBUMP* (Keegan & Winn, 2007) with *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A clear molecular-replacement solution was found using PDB entry 2oga (DesV from *S. venezuelae*, which shows 35% sequence identity to NtdA; Burgie *et al.*, 2007) as the search model. The rotation search, which was performed within the resolution range 28–2.3 Å, yielded two top solutions with Rf/σ of 7.3 and 6.0 (in contrast to 4.0 for the third solution), which confirmed the presence of two monomers per asymmetric unit. The best solution after the translation function had an R factor of 59%. Restrained refinement (30 cycles) of this solution with *REFMAC*5 (Murshudov *et al.*, 1997) as implemented as part of *MrBUMP* significantly lowered the R factors to R = 39.4% and R_{free} = 44.9% with a figure of merit of 0.50. Examination of the molecular-replacement solution using the program *Coot* (Emsley & Cowtan, 2004) showed no steric clashes or overlaps between the two molecules. The solution has a dimeric arrangement in the asymmetric unit. This is consistent with the other known sugar aminotransferase structures and is considered to be the physiologically active form of the protein. The initial $F_o - F_c$ electron-density map shows clear density for PLP (Fig. 3).

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