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# The *Bacillus anthracis* arylamine *N*-acetyltransferase ((BACAN)NAT1) that inactivates sulfamethoxazole, reveals unusual structural features compared with the other NAT isoenzymes ☆

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ABSTRACT

**Arylamine *N*-acetyltransferases (NATs) are xenobiotic-metabolizing enzymes that biotransform arylamine drugs. The *Bacillus anthracis* (BACAN)NAT1 enzyme affords increased resistance to the antibiotic sulfamethoxazole through its acetylation. We report the structure of (BACAN)NAT1. Unexpectedly, endogenous coenzymeA was present in the active site. The structure suggests that, contrary to the other prokaryotic NATs, (BACAN)NAT1 possesses a 14-residue insertion equivalent to the "mammalian insertion", a structural feature considered unique to mammalian NATs. Moreover, (BACAN)NAT1 structure shows marked differences in the mode of binding and location of coenzymeA when compared to the other NATs. This suggests that the mechanisms of cofactor recognition by NATs is more diverse than expected and supports the cofactor-binding site as being a unique subsite to target in drug design against bacterial NATs.**


## 1. Introduction

Arylamine *N*-acetyltransferases (NATs: EC 2.3.1.5) are a family of enzymes that catalyze the acetylation of aromatic amines using acetyl coenzyme A (AcCoA) [1,2]. In humans, NATs play an important role in the detoxification and/or bioactivation of numerous drugs and xenobiotics [3]. These enzymes are found in a range of eukaryotic and prokaryotic species [4–7] where they may play diverse roles [6]. Determination of the structures of NATs has helped to understand the catalytic mechanisms and functions of these enzymes [6]. So far, the crystal structures of the two human NAT isoenzymes and six prokaryotic NATs have been described [8–14]. Moreover, the NMR structure of the Syrian Hamster (MESAU)NAT2 has been reported [15]. These studies identified a common fold that comprises three domains and a cysteine protease-like catalytic triad [6,16]. Although mammalian and prokaryotic NATs share the same fold, differences at the amino acid levels are known to have functional consequences [6,10,12,13,15].

Although the role of NAT in prokaryotes remains unclear, these enzymes may contribute to adaptative and/or defense mechanisms towards environmental toxins present in the habitats of bacteria [6,17]. Certain bacterial NATs are able to acetylate various aromatic amine antibiotics and increasing evidence suggests that these enzymes could contribute to antibiotic resistance in bacteria such as *Mycobaterium tuberculosis* [18,19]. In *Bacillus anthracis*, the (BACAN)NAT1 isoenzyme (formerly known as BaNATC) acetylates efficiently sulfamethoxazole and affords higher than normal resistance to this antimicrobial when expressed in *Escherichia coli* [20].

Here we present the crystal structure of (BACAN)NAT1 in complex with the cofactor coenzymeA. This novel NAT structure shows marked differences in the mode of recognition and the location of the cofactor compared to other prokaryotic and mammalian NAT enzymes. Moreover, we show that (BACAN)NAT1 possesses a 14-residues insertion equivalent to the "mammalian loop" found in human isoenzymes. This loop is likely to contribute to the shape of the active site cleft and subsequently to the recognition of the cofactor by (BACAN)NAT1, as suggested for (HUMAN)NAT2 [12,13]. By demonstrating both unexpected structural similarities

*Abbreviations:* NAT, arylamine *N*-acetyltransferase; SMX, sulfamethoxazole; 3′ADP, 3′ adenosine diphosphate; 5AS, 5-aminosalycilic acid; AcCoA, acetyl Coenzyme A; CoA, coenzyme A; PPi, pyrophosphate

☆ The structure has been deposited in the Protein Data Bank (PDB code 3LNB).

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with mammalian NATs and important divergence in cofactor binding among different NAT enzymes, we provide a better understanding of the structures and functions of members within this important family of xenobiotic-metabolizing enzymes.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated all other reagents were from Sigma–Aldrich.

### 2.2. Expression and purification of (BACAN)NAT1

Recombinant (BACAN)NAT1 was expressed in E.coli BL21 (DE3) and purified using nickel resin as described previously [20]. Purified (BACAN)NAT1 was dialysed against 25 mM Tris–HCl pH 7.5, 1 mM EDTA and concentrated to 8 mg/ml using ultracentrifugation concentrators (Amicon) [26].

### 2.3. Crystallization and data collection

Crystallization trials were performed at 294 K with AmSO4 Suite (Qiagen) and CrystalQuick microplates (Greiner). We used the hanging-drop vapour-diffusion method by mixing 1.3 μl of concentrated protein solution with 0.7 μl of reservoir solution. Microcrystals were observed after two weeks with several solutions (No. 23, 31, 37, 45). Using a mix of 2 μl of protein with 1 μl of reservoir solution, the crystallization conditions were optimized on EasyXtal Tools X-Seal (Qiagen) with 1.8 M ammonium sulphate and 0.17 M potassium nitrate. Crystals, suitable for X-ray analysis, grow in 5-7 days at a maximal size of 120 × 120 × 50 μm. They belonged to space group $P4_12_12$. For cryoprotection, the crystal was fished from the drop, put directly into sodium formate (7 M), mounted in a cryoloop (Hampton Research) and immersed into liquid nitrogen prior to X-ray diffraction analysis.

Diffraction data were collected at 100 K on the beamline ID23-1 at the ESRF using a MAR CCD detector. We used an exposure time of 0.1 s with a transmission of 16%. One thirty images were collected with 1° oscillation per image at wavelength of 0.92 Å. The data were subsequently processed with XDS [21]. The structure of (BACAN)NAT1 protein was solved at 2 Å resolution by molecular replacement, using the coordinates of *Salmonella typhimurium* NAT (PDB entry:1E2T). The atomic coordinates and structure factors have been deposited in the PDB with the accession code 3LNB. The CCP4 Software Suite was used for the structure analysis [22].

### 2.4. High Performance Liquid Chromatography analysis

To detect the presence of CoA in the (BACAN)NAT1 samples used for crystallisation, the protein samples were deproteinized by perchlorate (100 μl of ice-cold aqueous perchlorate (15% w/v) added to 100 μl of protein sample) and centrifugated (5 min at 12,000×*g*). The molecules present in the supernatant (20 μl) were identified by reverse-phase separation on a C18 column and Photo-Diode Array (PDA) detection. Commercialy available CoA was used as a standard.

## 3. Results and discussion

### 3.1. Overall structure

The multiple sequence alignment of (BACAN)NAT1 with several structurally and functionally characterized NATs shows that (BACAN)NAT1 shares around 30% and 20% amino acid sequence identity with bacterial and human NAT enzymes, respectively (Figs. S1 and S2). (BACAN)NAT1 contains the characteristic conserved NAT functional motifs described in all NAT enzymes characterized so far [5,23]. Moreover, the alignments also suggests that the (BACAN)NAT1 possesses an insertion of 14 amino acids at a position equivalent to the 17 amino acid insertion loop (known as the "mammalian insertion") which is considered to be specific of mammalian NATs [12,15,23,24].

**Table 1**
Data-collection and refinement statistics for (BACAN)NAT1 crystal.

| | |
|---|---|
| *Crystallographic data quality* | |
| X-ray source | ID23-1, ESRF Grenoble |
| Wavelength (Å) | 0.92 |
| Temperature (K) | 100 |
| Unit-cell parameters (Å, °) | $a = b = 53.99$, $c = 172.44$<br>$\alpha = \beta = \gamma = 90$ |
| Space group | $P4_12_12$ |
| Resolution limits† (Å) | 50.0–2.0 (2.2–2.0) |
| Number of observations measured† | 17034 (3487) |
| $I/\sigma^{\dagger}(I)$ | 20.45 (7.91) |
| $R_{merge}$†,§ (%) | 6.3 (17.7) |
| Overall completeness†(%) | 94 (79.9) |
| *Refinement statistics* | |
| Number of non-hydrogen atoms (Protein/Water/CoA/Other) | 2055/137/48/6 |
| Resolution range (Å) | 45.8–2.0 |
| $R/R_{free}$ (%) | 20.3/24.4 |
| R.M.S.D. bonds (Å)/angles (°) | 0.06/1.3 |
| Average B value-all (Å$^2$) Protein/Water/CoA/Other | 18.85/23.68/20.26/25.06 |

† Values in parentheses refer to the highest resolution shell (2.2–2.0 Å).
§ $R_{merge} = \sum_h\sum_i|I_{hi}-\langle I_h\rangle|/\sum_h\sum_i I_{hi}$, were $I_{hi}$ is the i$^{th}$ observation of the reflection h, while $\langle I_h\rangle$ is the mean intensity of reflection h.

The (BACAN)NAT1 crystal structure was solved by molecular replacement using the *Salmonella typhimurium* NAT structure and refined to 2 Å (Table 1). Clear electron density was present from residues 1 to 167 and from residues 183 to 267. All residues but one (Ala127) were in allowed regions of the Ramachandran plot (data not shown). More importantly, an additional electron density was found and identified as being coenzyme A (CoA) (Fig. 1). The (BACAN)NAT1 structure resembles the overall fold of eukaryotic and prokaryotic NATs [23] (Fig. 1). It consists of three domains of similar size. Domain I is an α-helical bundle (amino acids 1–84). The second domain forms a β-barrel (amino acids 85–196). The structure of 15 residues (amino acids 168–182 which encompass the 14-residues insertion) within domain II could not be determined due to lack of electron density. Domains I and II are disposed in such a way that Cys70, His108 and Asp123 form the catalytic triad (Fig. 1) [8]. The geometry of the catalytic residues is conserved between (BACAN)NAT1 and the other NAT structures with a RMSD around 0.1 Å. Domain III forms an α/β lid (amino acids 198–267). This domain shows the most variation (both in length and sequence) from the other NATs (Fig. S1) [23]. The surface of these domains presents a deep and wide active-site pocket; at the base of which the catalytic cysteine residue is found (Fig. 1). The (BACAN)NAT1 structure differs from other bacterial enzymes by RMSD values (ranging from 1.3 Å) over 183 and 217 residues for (RHILO)NAT1 and (SALTY)NAT1, respectively. Although amino acid sequence identity between (BACAN)NAT1 and human NATs is only 20%, the structure of (BACAN)NAT1 displays RMSD values of 1.4 Å over 215 residues with these enzymes.

### 3.2. Presence of a "mammalian-like" insertion in (BACAN)NAT1

Mammalian NATs are known to possess a 17-residue insertion ("mammalian insertion") which forms a loop between two