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The role of lysine^{100} in the binding of acetylcoenzyme A to human arylamine N-acetyltransferase 1: Implications for other acetyltransferases

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ABSTRACT

The arylamine N-acetyltransferases (NATs) catalyze the acetylation of aromatic and heterocyclic amines as well as hydrazines. All proteins in this family of enzymes utilize acetyl coenzyme A (AcCoA) as an acetyl donor, which initially binds to the enzyme and transfers an acetyl group to an active site cysteine. Here, we have investigated the role of a highly conserved amino acid (Lys^{100}) in the enzymatic activity of human NAT1. Mutation of Lys^{100} to either a glutamine or a leucine significantly increased the K_a for AcCoA without changing the K_b for the acetyl acceptor p-aminobenzoic acid. In addition, substrate inhibition was more marked with the mutant enzymes. Steady state kinetic analyzes suggested that mutation of Lys^{100} to either leucine or glutamine resulted in a less stable enzyme–cofactor complex, which was not seen with a positively charged arginine at this position. When p-nitrophenylacetate was used as acetyl donor, no differences were seen between the wild-type and mutant enzymes because p-nitrophenylacetate is too small to interact with Lys^{100} when bound to the active site. Using 3′-dephospho-AcCoA as the acetyl donor, kinetic data confirmed that Ly^{100} interacts with the 3′-phosphoanion to stabilize the enzyme–cofactor complex. Mutation of Lys^{100} decreases the affinity of AcCoA for the protein and increases the rate of CoA release. Crystal structures of several other unrelated acetyltransferases show a lysine or arginine residue within 3 Å of the 3′-phosphoanion of AcCoA, suggesting that this mechanism for stabilizing the complex by the formation of a salt bridge may be widely applicable in nature.

1. Introduction

Acetyltransferases are a diverse superfamily of enzymes involved in the modification of small drug molecules, xenobiotics, peptide and proteins. They are found in all prokaryotic and eukaryotic species studied to date and are essential for numerous intracellular pathways. While the acetyl acceptor varies considerably between different acetyltransferases, they all share a common acetyl donor, acetylcoenzyme A (AcCoA). The arylamine N-acetyltransferases (NATs; EC 2.3.1.5) are xenobiotic metabolizing enzymes widely distributed in the animal kingdom [1]. They are distinguished by the presence of a conserved catalytic triad that prefers aromatic amine and hydrazine substrates [2]. In humans, there are 2 NATs (NAT1 and NAT2) and their crystal structure and catalytic function have been described in detail [3–6]. Both NAT1 and NAT2 are genetically polymorphic, which impacts on the pharmacology of many therapeutic agents that are metabolized by these enzymes [7]. Moreover, recent studies have shown a relationship between NAT1 and cancer cell proliferation and survival suggesting that this protein is a potential drug target [7,8]. There have also been a number of reports on the development of small molecule inhibitors for human and non-human NATs [9–12].

The NATs catalyze the acetylation of small molecules via a double displacement or ping pong bi bi reaction [13]. An in-depth understanding of the catalytic mechanism of the mammalian NAT's was provided by Wang et al. who examined the acetylation of various substrates by the hamster homolog of NAT1 using Bronsted plot analyses, kinetic solvent isotope effects and pH-dependence studies [14,15]. This work showed that the formation of a thiolate-imidazolium ion pair by Cys^{68} and His^{107} was essential for enzymatic function. The acetyl donor, which binds first, is orientated by several amino acids that line the cavity of the active site. This is true for both mammalian and bacterial NAT

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[6,16]. In human NAT2, the amino acids reside in the β2 and β3 domains, which extent from amino acids 93–104 (FYIPPVNKYSTG), and in the α9 domain at amino acids 208–217 (YLQTSPTSF). These regions are highly conserved across mammalian NATs suggesting a common mechanism for AcCoA binding (Fig. 1A).

The outer surface of the active site pocket for both human NAT1 and NAT2 contains a conserved lysine (Lys^{100}). The crystal structure of the NAT2-CoA binary complex shows that Lys^{100} is in close proximity to the 3′-phosphoanion of CoA (RCSB Protein Data Bank 2PFR) [6]. A similar arrangement is seen with the ε-amino group of Lys^{248} in the NAT homolog from *Bacillus anthracis* [16]. In the mammalian NATs, K^{100} is located on the flexible β2–β3 loop, which shifts toward the center of the active site cleft upon CoA binding [6]. This suggests that Lys^{100} may be involved in the interaction of the acetyl donor with the NATs.

NAT1 is widely distributed in the body and is responsible for metabolism of many therapeutic and carcinogenic compounds [17]. The crystal structure of NAT1 has been reported and it retains the same structural features as other mammalian NATs [6]. Site-directed mutagenesis has been extensively used with both NAT1 and NAT2 to discover critical amino acids involved in the reaction mechanism [15,18,19], substrate specificity [20] and stability [21,22]. Because recent studies have suggested that NAT1 may be a novel drug target [7,8], insight into how substrates interact with the protein provides important information for the design and development of small molecule inhibitors. In the present study, we have investigated the role of the conserved K^{100} in the acetylation of substrates by NAT1 using steady state enzyme kinetics of wild-type and K^{100} mutants that vary in the charge of their side chain. In addition, different acetyl donors have been studied with the view of establishing whether K^{100} influences their interaction with the enzyme.

2. Materials and methods

2.1. Materials

p-Aminobenzoic acid, acetylcoenzyme a, de-phospho-coenzyme A and acetic anhydride were obtained from Sigma–Aldrich (St Louis, USA). RPMI1640, serum and LipofectAMINE 2000 were obtained from Life Technologies (Victoria, Australia). Primers were purchased from GeneWorks (South Australia, Australia). All other chemicals were of analytical grade.

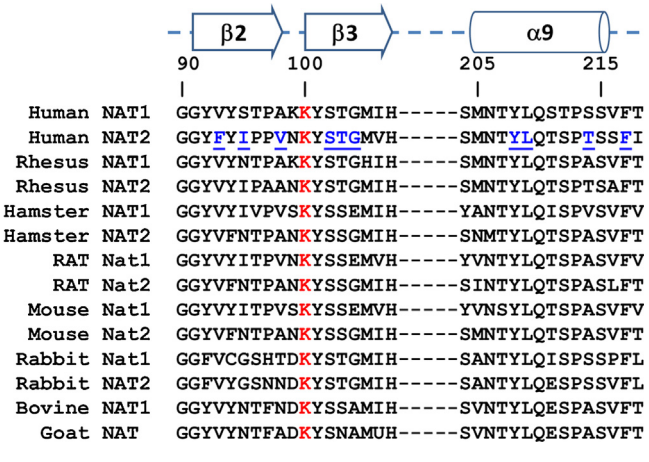

Fig. 1. Sequence homology of mammalian NATs in the β2–β3 and α9 region of the protein. Blue underlined letters refer to amino acids identified in the human NAT2 crystal structure that bind specific regions of the AcCoA molecule [6]. The conserved lysine at position 100 is shown in red. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

2.2. Cells culture

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Mutagenesis

Lys^{100} was mutated to either a glutamine, leucine or arginine residue using the GENEART site-directed mutagenesis system (Life Technologies, Vic., Australia) as described in the manufacturer's protocol. Wild-type FLAG-tagged human NAT1 [22] was used as template with the following primers: K^{100}Q FP, 5′-agcactccagccaaa-*caa*tacagcactggcatg-3′, K^{100}Q RP, 5′-catgccagtgctgta*ttg*tttggctg-gactgct-3′, K^{100}L FP, 5′-agcactccagccaaa*tta*tacagcactggcatg-3′, K^{100}L RP, 5′-catgccagtgctgta*aat*tttggctggactgct-3′, K^{100}R FP, 5′-agcactc-cagccaaa*aga*tacagcactggcatg-3′, K^{100}R RP,5′-catgccagtgctgta*tct* tttggctggactgct-3′. Clones were verified by sequencing.

2.4. Transient transfection and protein expression

Cells were seeded at a density of 0.8×10^6 cells/well in 6-well plates and allowed to adhere overnight. They were then transiently transfected with 4 μg plasmid DNA using LipofectAMINE 2000 according to the manufacturer's instructions and incubated overnight. Cells were washed twice with cold PBS and then scraped into 0.6 ml of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM dithiothreitol and disrupted on ice by sonication. Cell lysates were centrifuged at 16,000 × *g* for 10 min (4 °C) and the supernatants retained for FLAG Western blot and NAT1 activity assays.

2.5. NAT1 assay

NAT1 activity was assayed using p-aminobenzoic acid (PABA) as substrate and either AcCoA, dephospho-AcCoA, or p-nitrophenylacetate (pNPA) as cofactor. N-acetyl-PABA was measured by high performance liquid chromatography as previously described [23]. Kinetic parameters for PABA were determined using 1100 μM cofactor and 0–1200 μM PABA. For the determination of cofactor kinetic parameters, 420 μM PABA was used with 0–1200 μM cofactor. All reactions were performed under linear conditions with respect to substrate and protein. NAT1 activities were normalized for protein expression using FLAG Western blots of each cell lysate.

2.6. Synthesis of dephospho-AcCoA

Acetyl-3′-dephospho-coenzyme A was synthesized as previously described [22]. Briefly, 2.3 mg 3′-dephospho-coenzyme A (Sigma–Aldrich) was dissolved in 200 μl NH_4OH. Acetic anhydride (16 μl) was added on ice over 30 min with shaking. The solution was then freeze dried to remove the solvent and the excess acetic acid. The acetyl-3′-dephospho-coenzyme A was dissolved in water and analyzed by HPLC before use.

2.7. Data analysis

Steady state kinetics were analyzed based on the reaction mechanism shown in Fig. 2A, which describes a double displacement or ping pong bi bi reaction with substrate inhibition. The initial velocity (v) was described by:

$$v = \frac{V_{max} \cdot A \cdot B}{K_a \cdot B \cdot (1 + (B/K_i)) + K_b \cdot A + A \cdot B} \quad (1)$$