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Insights into how protein dynamics affects arylamine *N*-acetyltransferase catalysis

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Abstract

Arylamine *N*-acetyltransferases (NATs) detoxify arylamines and hydrazine xenobiotics by catalyzing their *N*-acetylation, which prevents their bioactivation. Here, we reveal how structural dynamics impact NAT protein function. Our data suggest that there are multiple conformations in the catalytic cavity of hamster NAT2 that exchange on the millisecond time scale and enable NATs to accommodate substrates of varying size. The regions spanning N177–L180 and D285–F288, which form unique structures in mammalian NATs, possess inherent motions on the nanosecond time scale. The latter segment becomes more restricted in its motions upon substrate binding according to our NMR XNOE data. This greater rigidity appears to stem from interactions with the substrate. Finally, NAT acetylation has been suggested to protect these enzymes from ubiquitination. Our NMR data on a catalytically active state of hamster NAT2 suggest that structural rearrangements caused by its acetylation might contribute to this protection.

Keywords

Arylamine *N*-acetyltransferase; backbone dynamics; XNOE; NMR

Introduction

Humans possess two arylamine *N*-acetyltransferases (NAT1 and NAT2), which catalyze acetylation of arylamines, arylhydrazines and *N*-hydroxylated metabolites through the transfer of an acetyl group from acetyl CoA [1;2]. The catalysis requires a strictly conserved cysteine residue that accepts the acetyl group of acetyl CoA to form an acetyl-enzyme intermediate [3]. This acetyl group is subsequently transferred to substrate and the acetylated product released. Crystal structures of NAT from *Salmonella typhimurium* [4] and *Mycobacterium smegmatis* [5] revealed this cysteine to be part of a strictly conserved (Cys-His-Asp) catalytic triad. NAT activity is important because it is a major determinant of whether arylamine and arylhydroxylamine carcinogens are activated or detoxified [6].

Genetic variations exist between individuals that result in proteins with altered enzymatic activity and cellular abundance. Epidemiological studies have connected the “slow acetylator

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phenotype” to specific cancer types, the most well-established being bladder cancer [7;8;9]. Certain NAT polymorphisms with reduced cellular activity have been demonstrated to undergo constitutive ubiquitination and in turn degradation by the 26S proteasome [10;11]. In addition, the ubiquitin-proteasome pathway is proposed to play a general regulatory role in NAT1 catalysis, as acetylated NAT is stable in cells, whereas its non-acetylated form is rapidly ubiquitinated and in turn degraded [10].

An NMR-based structural model of hamster NAT2 was reported in 2006 [12] and the unique structural features of mammalian NATs further revealed by crystal structure of human NAT1 (F125S) [13]. Hamster NAT2 shares 82% sequence identity (Fig. 1A) and identical substrate specificity with human NAT1 [14]. Substrates for human NAT1 and hamster NAT2 include the carcinogens arylamine 2-aminofluorene [15;16] and 4-aminobiphenyl [16], *p*-aminobenzoic acid (PABA) [15], and *p*-aminobenzoylglutamic acid, a folic acid metabolite and likely endogenous substrate [17]. Here, we characterize the intrinsic dynamical properties of hamster NAT2 by NMR to explore its role in NAT substrate binding and catalysis. In addition, we determine how acetylation impacts the spectral properties of hamster NAT2 to conclude that changes in tertiary structure occur in NAT during catalysis.

Materials and methods

Sample preparation and NMR experiments

Hamster NAT2 was expressed and purified from *Escherichia coli* for NMR analysis as described previously [12]. Acetylated hamster NAT2 was prepared by adding 10 times molar excess acetyl CoA to a sample containing 300 μ l of ^{15}N labeled hamster NAT2 on ice. To trap hamster NAT2 in a catalytically active, intermediate state, the sample was kept on ice until the NMR experiment. ^1H , ^{15}N heteronuclear NOE enhancements (XNOE) as well as T1 relaxation experiments were recorded with ^{15}N , 60% ^2H labeled hamster NAT2 protein (0.4 mM) without or with 20 molar excess of its substrate PABA in NMR buffer 1 (30 mM sodium phosphate, 50 mM NaCl, 4 mM DTT, 1 mM EDTA, 0.1% NaN_3 , pH 6.8) on a Varian 800 MHz spectrometer equipped with a cryogenically cooled probe and at 10 $^\circ\text{C}$. The ^1H , ^{15}N HSQC spectra of non-acetylated or acetylated hamster NAT2 were acquired at a sample concentration of 0.3 mM in NMR buffer 2 (75 mM sodium phosphate, 100 mM NaCl, 4 mM DTT, 1 mM EDTA, 0.1% NaN_3 , pH 6.8) on an 800 MHz spectrometer equipped with a conventional probe and at 5 $^\circ\text{C}$. Data processing and analysis were performed by using NMRPipe [18] and XEASY [19], respectively.

NMR dynamic studies

Rates for ^{15}N longitudinal $R_N(N_Z)$ was derived by fitting data acquired with different relaxation delays to a single-exponential decay function, and error values were determined by repeating one data point. Two spectra were recorded for steady-state NOE intensities, one with 4 seconds of proton saturation to achieve the steady-state intensity and the other as a control spectrum with no saturation to obtain the Zeeman intensity. The control spectrum was repeated to determine error values. Heteronuclear NOE enhancements (XNOE) were then calculated from the ratio described in Equation 1, as described in [20].

$$\text{XNOE} = \left| \left(\frac{\gamma_{\text{H}}}{\gamma_{\text{N}}} \right) \left[\frac{R_N(\text{H}_Z^{\text{N}} \leftrightarrow \text{N}_Z)}{R_N(\text{N}_Z)} \right] \right| \quad (1)$$

Residues were designated as highly flexible with fast internal motions if their XNOE magnitudes were at least 1.5 times the standard deviation (SD) above the average; flexible if

their value were larger than the average value, but smaller than 1.5*SD above the average; or restricted if their values were smaller than the average value.

Hamster NAT2 modeling

The published crystal structure of human NAT1 (F125S) [13] was used (PDB ID: 2IJA) to generate a homology-based model of hamster NAT2. Modeler of INSIGHT II (Accelrys) was used for this purpose.

Hamster NAT2/PABA complex model building

The hamster NAT2:PABA complex was generated by using HADDOCK1.3 (*High Ambiguity Driven protein-protein DOCKing*) [21] in combination with CNS [22], as described previously [12]. The lowest energy model structure was used for figures and analysis.

Results and discussion

The loop covering the NAT catalytic cavity is flexible in free hamster NAT2

In a previous study, we assigned chemical shift values to the backbone atoms of hamster NAT2 by using modern NMR techniques [12]. We were able to unambiguously assign 92% of the amide resonances for hamster NAT2 as well as all of the crosspeaks that appear in this protein's ^1H , ^{15}N HSQC spectrum. All unassigned amide atoms were entirely absent from the spectra, as described below. To probe the internal dynamics of hamster NAT2, we first performed NMR relaxation experiments to measure amide transverse ($R_N(N_X)$) and longitudinal ($R_N(N_Z)$) relaxation rates. However, fast amide transverse relaxation rates made it impossible to obtain reliable ($R_N(N_X)$) values. Therefore, amide heteronuclear NOE enhancement (XNOE) experiments were done to detect XNOE values on hamster NAT2 in its free non-acetylated state as well as in its substrate-bound state. XNOE experiments probe high frequency internal dynamics (on the picosecond to nanosecond time scale), and this experiment can be used to identify flexible regions within a protein [23].

The assigned residues of hamster NAT2 were partitioned as highly flexible, flexible or restricted based on their relative XNOE values, as described in Materials and methods, with an average value for all residues of 0.22 (Table 1). This analysis revealed the N-terminus, G11 - D20, K141 - D142, N156 - E157, and L275 - L279 to be highly flexible (displayed in green in Fig. 1B). The flexibility of these regions is also reflected in their fast T1 relaxation rates (Supplementary Fig. 1). Importantly, the region spanning L275 - L279 is part of a loop structure that extends across the catalytic cavity and restricts its accessibility [13]. By contrast, in prokaryotic NAT species, the analogous C-terminal residues are peripherally located and remote from the catalytic cavity [4]. The change in the positioning of the C-terminal region can be partially attributed to extensive interactions between its amino acid constituents and residues of an insertion region spanning E167 - K183, which is entirely absent in prokaryotic NATs. In addition to van der Waals interactions, hydrogen bonds are formed between the sidechains of D179 and H283 and the backbone carboxyl oxygen of L180 and amide nitrogen of I290. Altogether, these two structural regions in mammalian NATs produce an additional "gate-like" structure that is not present in prokaryotic NAT structures to ultimately yield a more restricted catalytic cavity. These two regions are highlighted in yellow in Fig. 1C.

Residues N177 - L180 and D285 - F288 restrict accessibility to the catalytic cavity, but exhibit larger XNOE values than the average value. This comparison indicates that they are relatively flexible with intrinsic dynamics characterized by high frequency motions (Fig. 1C). We propose that the inherent flexibility of this region serves to promote substrate interaction, which would be inhibited if this loop were rigidly docked into the core of the protein, as discussed below.

The loop covering NAT's catalytic cavity becomes more rigid after substrate binding

In previous research, we demonstrated that hamster NAT2 binds its substrate PABA, even when it is not acetylated and produced a model structure of the hamster NAT2:PABA complex [12]. We determined XNOE values (Fig. 2A) as well as longitudinal relaxation rates for PABA-bound hamster NAT2 to afford insight into how substrate binding affects the protein's dynamic properties. Whereas the average XNOE enhancement and $R_N(N_Z)$ relaxation values are comparable for substrate-bound and free hamster NAT2 (Table 1), the XNOEs of D285, R286, F287 and F288 decrease from 0.32, 0.23, 0.35 and 0.21 to 0.21, 0.17, 0.17 and 0.11, respectively (Table 2). These changes indicate that these residues become less flexible, which we propose is caused by their direct interaction with PABA. Indeed, in our hamster NAT2:PABA model structure, the aromatic carbons of F287 and F288 are proximal to PABA's aromatic ring (Fig. 2B).

Backbone dynamics of hamster NAT2 suggest that its catalytic cavity undergoes conformation exchange on the millisecond time scale

Despite being able to assign the amide resonances of 258 hamster NAT2 amino acids, those of ten within the catalytic core (C68, Y76, W77, T80, G126, R127, S128, M131, W132 and E133) were severely broadened and missing from the ^1H , ^{15}N HSQC spectrum (Fig. 2C). Their absence from the NMR spectra suggests that the catalytic pocket region of hamster NAT2 undergoes conformational exchange in the millisecond time scale (as the data is acquired within 100 ms) when the protein is not acetylated or substrate bound. Consistent with this hypothesis, observable residues within this region exhibited larger linewidths compared to other NAT resonances (Fig. 2D).

The hypothesis that slower, low frequency motions dominate the dynamics of the catalytic cavity was further demonstrated by the NMR relaxation data. Specifically, the small XNOE enhancements for most of the observable residues in the α -helix spanning C68 - M82 and the region spanning D122 - P134, which adopts a loop with an intervening 3^{10} -helix, were exhibited (Fig. 1C). These regions include C68 and D122 of the catalytic triad (Fig. 2C). Based on our NMR data, we propose that multiple conformations exist in the catalytic pocket region of hamster NAT2. It is possible that a population of higher energy species adopts a conformation that is amenable to the next step of the catalytic cycle. There is precedent for such use of intrinsic dynamics in enzyme catalysis. In particular, the existence of dynamics and higher energy states partially contribute to the high enzymatic efficiency of dihydrofolate reductase [24]. This model is appealing for NATs, as the presence of chemical exchange between different structural states would also have implications for its ability to acetylate a broad range of substrates [25]. Namely, the catalytic cavity could more easily accommodate substrates of varying size were it not rigidly defined.

Effects of acetyl CoA on hamster NAT2

To investigate how acetylation affects hamster NAT2 structure and dynamics, we acquired an ^1H , ^{15}N HSQC spectrum on hamster NAT2 incubated with 10-fold molar excess acetyl CoA (Fig. 3A, red). Since the acetyl group of acetylated NAT is hydrolyzed at room temperature [26;27] but not at 0°C [27], the experiment was performed at 5°C. Whereas no spectral changes were observed due to the temperature change (Fig. 3B), the addition of acetyl CoA caused specific crosspeaks to attenuate (Fig. 3A). To test whether the resulting NAT species represents an enzymatically active state, we added PABA to the mixture at equimolar concentration with acetyl CoA. ESI-TOF mass spectrometry experiments revealed that all of the PABA in this sample was acetylated, whereas that in the control experiment conducted with PABA in the presence of acetyl CoA and no hamster NAT2 was not (Fig. 3C). Therefore, we concluded that the NAT species observed was either acetylated or some other intermediate along the pathway of NAT acetylation.

The addition of acetyl CoA caused a large number of amide resonances to attenuate or shift, including G65, G66, L69, Q70, N72, H73, L75, V93, F94, N95, S102, S103, G104, I106, L108, V121, F125, Q130, Q163, F192, S215, V216, F217, K220, S224, L225, F287 and F288 (Fig. 3A). As noted earlier, attenuation is caused by conformational exchange during data acquisition. Although the affected residues map to the catalytic cavity, we were surprised by how many were affected given the small size of an acetyl group. We therefore tested whether the spectral changes were in part due to interaction with CoA by adding it to hamster NAT2.

Non-acetylated CoA was incubated at 10-fold molar excess with ^{15}N labeled hamster NAT2, which was monitored by a ^1H , ^{15}N HSQC experiment. A comparison with free hamster NAT2 demonstrated that the amide resonances of L69, H73, V93, F94, S102, S103, G104, I106, Q130, S215, V216, K220, F287 and F288 attenuate and/or shift upon CoA binding (Fig. 4A and 4B). This finding is consistent with the human NAT2:CoA crystal structure, which revealed F93, S102, T103, G104, S216, S287 and L288 to bind CoA [13].

Importantly, a comparison of the spectral changes that result from CoA versus acetyl CoA addition revealed specific effects for certain NAT residues. In particular, attenuation and/or shifting of the amide resonances of G65, G66, Q70, N72, L75, N95, L108, V121, F125, Q163, F192, F217, S224 and L225 occurred upon acetyl CoA, but not CoA addition (Fig. 4A, 4B). Therefore, we conclude that NAT acetylation causes structural changes in the catalytic cavity that affect residues that are up to 18 Å away from the acetylation site, namely Sy of C68 (Fig. 4C). This finding is supported by previously published data indicating that NAT acetylation is protective against NAT ubiquitination [10]. Protein ubiquitination is highly regulated and substrate typically recognized via an E3 ubiquitin ligase protein, reviewed in [28]. Therefore, for acetylation to protect NAT from ubiquitination, there must be resulting changes to a protein interaction surface. Our data demonstrate acetyl CoA to affect residues that are not directly involved in catalysis, which are up to 18 Å away from the site of acetylation. We propose that the observed spectral changes are caused by structural rearrangements, which could provide an explanation for how acetylation blocks NAT ubiquitination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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hamster NAT2 are revealed by magnitudes of heteronuclear NOE enhancements. The average value and the value at $1.5 \times \text{SD}$ plus the average value are indicated by red dotted and dashed lines, respectively. The residues spanning C68 – M82 and D122 – P134 are colored in cyan, and the region spanning E167 to K185 and the C-terminal fragment G272 – T289 are highlighted in yellow.

Fig. 2.

(A) The backbone dynamic properties of hamster NAT2 bound to its substrate PABA are revealed by heteronuclear NOE enhancements. The average value and the value at 1.5*SD plus the average value are indicated by red dotted and dashed lines, respectively. (B) Expanded view of the catalytic cavity of hamster NAT2 in its PABA-bound state. Carbon, nitrogen, oxygen, and hydrogen atoms of the substrate are colored in green, blue, red, and white, respectively. The side-chain heavy atoms of F287 and F288 are colored in blue, while the catalytic triad (C68, H107, and D122) is highlighted in yellow. The distance between an aromatic atom of F288 and that of the substrate is provided. (C) Ribbon diagram of hamster NAT2 model structure based on crystal structure of human NAT1 (F125S) (PDB ID: 2IJA).

The catalytic triad (C68, H107, and D122) is highlighted in red, and the residues of the catalytic cavity that are severely broadening and missing from the ^1H , ^{15}N HSQC spectrum are displayed in red, while the catalytic region, which undergoes slow conformation exchange in solution, is highlighted in cyan. (D) 1D traces from ^1H , ^{15}N HSQC spectrum of hamster NAT2 for residues M1, H73 and D122. H73 and D122 are in hamster NAT2's catalytic cavity.

Fig. 3.

(A) Superposition of ^1H , ^{15}N HSQC spectra of hamster NAT2 without (black) and with acetyl CoA (red, molar ratio of 1:10 hamster NAT2 to acetyl CoA) reveals spectral changes. Significantly affected resonances are labeled. (B) Superimposed ^1H , ^{15}N HSQC spectra of NAT2 at 10°C (black) and 5°C (red). Only one peak, derived from N14, is observed to shift due to the change in temperature. (C) Addition of PABA to the hamster NAT2 mixed with acetyl CoA causes PABA to be acetylated. ESI-TOF spectra of PABA alone (upper panel) and with hamster NAT2, acetyl CoA, and PABA at 1:10:10 molar ratio (lower panel). This figure demonstrates that the PABA in sample is acetylated. A control experiment with acetyl CoA at

equimolar concentration with PABA and no hamster NAT2 present revealed the presence of only the non-acetylated PABA species (MW of 136.0).

Fig. 4.

(A) Superposition of expanded regions of ^1H , ^{15}N HSQC spectra of hamster NAT2 without (black) or with 10-fold molar excess acetyl CoA (red) or CoA (cyan) reveals residues specifically affected by the acetyl group. Significantly affected resonances upon CoA and/or acetyl CoA addition are labeled in red if they are only affected by acetyl CoA addition and black if they are affected by both. (B) Stereoview of hamster NAT2 with the residues affected by only acetyl CoA or both acetyl CoA and CoA binding are colored in green or blue, respectively. The catalytic triad is displayed in red. (C) Expanded view of (B) with identical coloring. The distance between $\text{S}\gamma$ of C68 and Q163's backbone amide proton is 18 Å as labeled.

Table 1

Average values of ^{15}N longitudinal $R_N(N_Z)$ relaxation rates and magnitudes of the heteronuclear NOE enhancements for hamster NAT2 alone and bound to PABA.

	XNOE	$R_N(N_Z)$ (s^{-1})
Free	0.22	0.51
PABA-bound	0.23	0.56

Table 2
Comparison of XNOE values for selected residues of hamster NAT2 alone and bound to PABA.

Residue Number	1	15	157	275	285	286	287	288
XNOE (free)	0.58	0.61	0.55	0.54	0.32	0.23	0.35	0.21
XNOE (PABA-bound)	0.57	0.60	0.55	0.65	0.21	0.17	0.17	0.11