Investigation of the Roles of Catalytic Residues in Serotonin N-Acetyltransferase*

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Serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase (AANAT)) is a critical enzyme in the light-mediated regulation of melatonin production and circadian rhythm. It is a member of the GNAT (GCN-5-related N-acetyltransferase) superfamily of enzymes, which catalyze a diverse array of biologically important acetyl transfer reactions from antibiotic resistance to chromatin remodeling. In this study, we probed the functional properties of two histidines (His-120 and His-122) and a tyrosine (Tyr-168) postulated to be important in the mechanism of AANAT based on prior x-ray structural and biochemical studies. Using a combination of steady-state kinetic measurements of microviscosity effects and pH dependence on the H122Q, H120Q, and H120Q/H122Q AANAT mutants, we show that His-122 (with an apparent pKₐ of 7.3) contributes ~6-fold to the acetyltransferase chemical step as either a remote catalytic base or hydrogen bond donor. Furthermore, His-120 and His-122 appear to contribute redundantly to this function. By analysis of the Y168F AANAT mutant, it was demonstrated that Tyr-168 contributes ~150-fold to the acetyltransferase chemical step and is responsible for the basic limb of the pH-rate profile with an apparent (subnormal) pKₐ of 8.5. Paradoxically, Y168F AANAT showed 10-fold enhanced apparent affinity for acetyl-CoA despite the loss of a hydrogen bond between the Tyr phenol and the CoA sulfur atom. The X-ray crystal structure of Y168F AANAT bound to a bisubstrate analog inhibitor showed no significant structural perturbation of the enzyme compared with the wild-type complex, but revealed the loss of dual inhibitor conformations present in the wild-type complex. Taken together with kinetic measurements, these crystallographic studies allow us to propose the relevant structural conformations related to the distinct alkylation and acetyltransferase reactions catalyzed by AANAT. These findings have significant implications for understanding GNAT catalysis and the design of potent and selective inhibitors.

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced in the brain by the pineal gland and controls behavioral and physiological circadian rhythms. The production of this hormone is dependent on the enzyme serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase (AANAT)), which fluctuates in response to light and dark signals and is the rate-limiting enzyme in the biosynthesis pathway (see Fig. 1A) (1). Concurrent with the fluctuation in AANAT, the level of circulating melatonin is high at night and low during the day (1). Upon sudden exposure to light in the middle of the night, levels of melatonin and AANAT decrease rapidly with a half-life of ~3.5 min (2).

AANAT is a member of the GNAT (GCN-5-related N-acetyltransferase) superfamily of enzymes, the other members of which include GCN-5, PCAF (p300/CBP-associated factor), and the aminoglycoside N-acetyltransferases. This superfamily has members found in all kingdoms of life and is characterized by a common substrate, acetyl-CoA, and a structural fold where acetyl-CoA binds (3). The primary sequences of these enzymes are poorly conserved, reflecting their ability to acetylate a variety of different substrates from histones to aminoglycoside antibiotics (3–5).

Previous work has shown that AANAT follows an ordered Bi Bi ternary complex mechanism in its catalysis of acetyl transfer, with acetyl-CoA binding to the enzyme first, followed by the binding of serotonin or tryptamine (6). Alternative substrates and pH-rate studies on AANAT revealed that ionizability of two or more groups, a potential catalytic base and acid, may be important in the reaction mechanism (7). Using the knowledge that both substrates bind to the enzyme simultaneously, a potent inhibitor (Kᵢ = 50 nM) was developed that incorporates both acetyl-CoA and serotonin (Fig. 1B) (8). Moreover, it was shown that AANAT itself can catalyze the formation of the bisubstrate analog via a newly discovered “alkylation transferase activity” (Fig. 1B) (9, 10). This activity, catalyzed in an apparently subtly altered form of the acetyltransferase active site (9, 10), allows for the delivery of a cell permeable “prodrug” form of the inhibitor.

The bisubstrate analog inhibitor has been used in complex with AANAT to obtain high resolution crystal structures of the enzyme (11, 12). These crystal structures show possible residues that may play a role in catalysis (Fig. 2). Based on these structural studies and preliminary kinetic analysis, Tyr-168, His-120, and His-122 have been proposed to be catalytic residues (11).

In this work, we examined the mechanistic function of these three residues in greater detail to attempt to identify more definitive roles for these residues in AANAT catalysis. Not only

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The abbreviations used are: AANAT, arylalkylamine N-acetyltransferase (serotonin N-acetyltransferase); MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; EPPS, 2-(N-cyclohexylamino)ethanesulfonic acid; GST, glutathione S-transferase; r.m.s.d., root mean square difference.

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should this knowledge be useful in relating AANAT to other GNAT family members, it may also provide clues to the development of specific inhibitors of AANAT that might be used to treat mood and sleep disorders (13).

EXPERIMENTAL PROCEDURES

General—Tryptamine, N⁵-methyltryptamine, racemic α-methyltryptamine, 5,5'-dithiobis(2-nitrobenzoic acid), glutathione-agarose, co-enzyme A, MES, MOPS, EPFS, and CHES were acquired from Sigma. Acetyl-CoA was purchased from Amersham Biosciences. Sucrose was purchased from Bio-Rad. N-Bromoacetyltryptamine (Fig. 1B, compound 1) and the bisubstrate analog (compound 2) were synthesized as previously described (8).

Preparation of AANAT Mutants—All AANAT mutants were prepared by PCR-based site-directed mutagenesis and purified as described previously (6), with several exceptions. Protein-expressing E. coli strains were grown as previously described, except that inductions with isopropyl-β-D-thiogalactopyranoside were performed at 16 °C for 22 h to enhance production of soluble protein. The GST-mutant fusion proteins were purified over a glutathione-agarose column after lysis by French press. The column (swelled from 20 to 50 ml) was equilibrated with 100 ml of lysis buffer prior to addition of the cleared lysate. The column was subsequently washed with 50 ml of lysis buffer and 200 mM NaCl, and the mutant proteins were eluted with 50 ml of lysis buffer, 110 mM NaCl, and 50 mM glutathione (pH 7.0) and collected in 10-ml fractions. Purity of the mutant enzymes had linear activities at each of the varied sucrose solutions, with the exception of H120A and H122A, for which the Km values were measured at the high and low points only and were not significantly altered in response to these changing sucrose levels.

Viscosity Studies on AANAT Mutants—All viscosity assays were performed in the same buffer and under the same conditions as described above. The sucrose solutions and relative viscosities were the same as described (7). Tryptamine Km values were assayed at each of the varied sucrose solutions, with the exception of H120A and H122A, for which the Km values were measured at the high and low points only and were not significantly altered in response to these changing sucrose levels.

Acetyltransferase Assays of AANAT Mutants—The acetyltransferase activity of the GST fusion proteins was measured using a non-continuous spectrophotometric assay involving the liberation of a colored product from the reaction of CoASH with 5,5'-dithiobis(2-nitrobenzoic acid) that was described previously (6). Assays were performed in a final volume of 0.3 ml in buffer consisting of 0.05 M sodium phosphate (pH 6.8), 0.5 mM NaCl, 2 mM EDTA, and 0.05 mg/ml bovine serum albumin at 30 °C. Varying amounts of acetyl-CoA (0.025–10 mM) and tryptamine (0.2–30 mM) were added to the reaction mixture, and reactions were initiated with enzyme diluted in the buffer described above, with final enzyme concentrations ranging from 0.05 to 1 μM, depending upon the mutant. Reactions were quenched with a guanidinium solution (0.3 M; 3.2 mM guanidinium chloride in 0.1 M sodium phosphate (pH 6.8)), and 0.05 ml of 5,5'-dithiobis(2-nitrobenzoic acid) (2 mM) was added prior to taking readings at 412 nm. Reaction rates were measured under initial conditions (linear with respect to time and enzyme concentration, <10% turnover of the limiting substrate). Assays involving Km measurements were conducted with a near-saturating concentration (Km > 3) of the unvaried substrate.

$$k_{cat}(control)/k_{cat}(viscogen) = k_2/(k_2 + k_3^0) \text{ (Eq. 1)}$$

where $k_3^0$ is the rate constant for the chemical step and $k_0$ represents the rate constant for product release in the absence of viscogen, as in previous studies (7). This equation assumes the formation of a fully saturated ternary complex, followed by the chemical reaction and then product release.

pH-rate Analysis of AANAT Mutants—Measurements of $k_{cat}$ and tryptamine Km were performed as described above and, under the conditions previously reported (7), over a pH range of 6.0–8.5. The buffers used in these reactions were as follows: MES (pH 6.0–6.7), MOPS (pH 6.7–7.6), EPFS (pH 7.7–8.5), and CHES (pH 8.3–8.5). All mutant enzymes had linear activities at each pH within the time used.
for the assays, and all were found to exhibit Michaelis-Menten kinetics at these pH values. Curve fits for $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ data were calculated as described previously (7).

Bisubstrate Analog Inhibition Assays—These were carried out with a range of inhibitor (compound 2) concentrations as described (9) using a Dixon analysis to give an apparent $K_i$ assuming a competitive inhibition model.

Alkyltransferase Activity of Y168F AANAT—Assays for alkyltransferase activity were carried out as described previously (9, 10). Briefly, the reaction conditions contained 50 mM sodium phosphate (pH 6.8), 0.05 mg/ml bovine serum albumin, 2 mM EDTA, and 500 mM NaCl at 30 °C. The products were separated and quantified by reversed-phase high pressure liquid chromatography and UV detection using the authentic materials as standards (9, 10). The $K_m$ for $N$-bromoacetyltryptamine (compound 1) was determined using a range of 0.025–2.5 mM in the presence of a constant and near-saturating concentration of CoASH (1 mM). The measurement of the $K_m$ for CoASH employed a concentration range of 0.025–5.0 mM with fixed and saturating $N$-bromoacetyltryptamine (compound 1; 2 mM). The final enzyme concentrations ranged from 3.5 to 20 μM; and in all cases, the limiting substrate was at least 5-fold higher than the enzyme concentration. In all cases, the background (nonenzymatic) rates were subtracted from the rate in the presence of enzyme to give the net enzymatic rate. Reaction rates were measured under initial conditions (linear with respect to time and enzyme concentration, <10% turnover of the limiting substrate).

X-ray Crystallographic Studies on Y168F AANAT—GST-AANAT Y168F protein was proteolytically cleaved of GST and purified over glutathione- and CoA-agarose resins as described previously (9). Crystals of the Y168F AANAT mutant bound to compound 2 were obtained using previously published procedures (12): space group C2221; unit cell dimensions $a = 53, b = 69, c = 89$ Å; and one enzyme-inhibitor complex/asymmetric unit. Cocrystals were cryoprotected with 100 mM MES (pH 6.5), 30% polyethylene glycol 2000, 100 mM Mg(OAc)$_2$, 2% (v/v) 2,4-methylpentanediol, 20% glycerol, 30 mM dithiothreitol, 20 mM spermidine, and 100 mM LiCl and frozen by immersion in liquid propane. Diffraction data were collected under standard cryogenic conditions on Beamline X4A (National Synchrotron Light Source, Brookhaven National Laboratory). Following data processing with

![X-ray structure of AANAT bound to the bisubstrate analog (compound 2). Tyr-168, His-120, and His-122 are highlighted and were the focus of the mutational studies detailed in this report.](image)

### Table I

Steady-state kinetic values for AANAT and AANAT mutants in acetyltransferase

| AANAT form         | $k_{\text{cat}}$ | Acetyl-CoA $K_m$ | Tryptamine $K_m$ |
|-------------------|-----------------|-----------------|-----------------|
|                   | (s$^{-1}$)      | (mM)            | (mM)            |
| Wild-type$^a$     | 25 ± 1          | 0.29 ± 0.013    | 0.17 ± 0.014    |
| Y168F             | 0.75 ± 0.04     | 0.03 ± 0.02     | 2.3 ± 0.32      |
| H120Q             | 24.5 ± 0.70     | 0.67 ± 0.03     | 1.2 ± 0.11      |
| H122Q             | 18.1 ± 1.11     | 0.791 ± 0.08    | 4.78 ± 0.62     |
| H120Q/H122Q       | 3.1 ± 0.07      | 1.11 ± 0.14     | 10.9 ± 0.63     |
| H120A             | 5.6 ± 0.15      | 1.3 ± 0.11      | 7.8 ± 0.59      |
| H122A             | 8.9 ± 0.08      | 0.86 ± 0.06     | 2.8 ± 0.09      |
| H120A/H122A       | 0.094 ± 0.003   | 7.6 ± 0.60      | 4.6 ± 0.40      |
| H120E             | 0.04$^b$        | 1.5 ± 0.20      | 3.0 ± 0.15      |
| H122E             | 2.1 ± 0.03      |                 |                 |

$^a$ Taken from Ref. 6.
$^b$ Approximate due to the instability of the enzyme.
DENZO/SCALEPACK (14), initial phases were obtained with our structure of the wild-type enzyme bound to compound 2 (Protein Data Bank code 1LOC). Rigid body refinement and Powell minimization with CNS (15) yielded clear evidence for the absence of the Tyr hydroxyl group at position 168 in an (Fo-Fc) difference Fourier synthesis (data not shown). The current 2.3-A refinement model for the structure contains residues 30–195, compound 2 in one conformation, and 90 water molecules, giving an R-factor of 19.3% and a free R-value of 24.6% with excellent stereochemistry (16) (overall G-value = 0.3; see Table III for data collection and refinement statistics).

RESULTS

Steady-state Kinetic Measurements for Y168F AANAT—Based on the x-ray structure of AANAT bound to compound 2, Tyr-168 appears to make a hydrogen bond with the CoA sulfur atom (11, 12). Tyr-168 was mutated to phenylalanine by site-directed mutagenesis to prevent the side chain from being able to participate in conventional hydrogen bonding or proton donation. This Y168F AANAT protein was expressed and purified in a manner similar to the wild-type enzyme. Interestingly, attempts at expressing Y168A AANAT were unsuccessful, presumably due to the instability of this protein. However, the Y168F AANAT mutant displayed catalytic activity that was linear with time and followed Michaelis-Menten kinetics, suggesting that it was sufficiently stable for kinetic studies. Kinetic analysis of Y168F AANAT showed a 30-fold lower kcat compared with wild-type AANAT and altered Km values for both substrates (Table I). The Km value for acetyl-CoA decreased to 0.03 mM, whereas the tryptamine Km rose to 2.3 mM.

To further understand the significance of these changes, analysis of the effects of the microviscogen sucrose on the kinetic parameters was performed. With appropriate controls, measurement of viscosity effects can be useful in determining the rate-limiting step of enzyme reactions because they can affect the diffusional processes, but not the chemical step (17). Previous viscosity studies (kcat/control/kcat(viscogen) versus relative viscosity) have shown that the rate-limiting step of AANAT when tryptamine is used as a substrate is predominantly a diffusion-controlled process (slope = +0.75, theoretical maximum = 1.0), most likely product (CoASH) release (7). In contrast, the chemical step of the reaction is rate-limiting when a “poor substrate” such as N'-methyltryptamine is substituted for tryptamine (slope = +0.10) (7). Poor substrates are often used as a control to detect any nonspecific effects of the viscogen on the enzyme (18). No detectable activity was seen with Y168F AANAT and N'-methyltryptamine as a substrate. However, we showed that α-methyltryptamine behaves as a poor substrate with Y168F AANAT, with kcat = 0.013 s⁻¹ and Km = 4.2 mM, whereas with wild-type AANAT, α-methyltryptamine shows a kcat similar to the tryptamine reaction (7).

The slope of the kcat/control/kcat(viscogen) versus relative viscosity plot for Y168F was found to be +0.38 using tryptamine as a substrate and +0.03 with α-methyltryptamine (Fig. 3). As expected with a poor substrate, the slope was virtually zero, indicating that the chemical step is indeed rate-limiting in this case. Despite the 30-fold lower kcat compared with the wild-type enzyme, the non-zero viscosity effect seen with tryptamine suggests unexpectedly that the chemical step is not fully rate-limiting in acetyl transfer catalyzed by this mutant. Thus, both the product release step and the chemical step are slowed by this mutation. Given the observation that the Km for acetyl-CoA is 10-fold lower with this mutant and the data described above, these results suggest that CoASH has a higher affinity for Y168F and is more slowly released.

The removal of an ionizable amino acid residue important in catalysis could result in a change in the pH-rate profile of an enzyme. The profile of log(kcat/Km) versus pH for wild-type AANAT from previous work (7) shows that there is an acidic limb reflecting one ionizable group with a pKa of 7.3 and a basic limb reflecting one ionizable group with a pKa of 8.5 (Fig. 4A). The pH-rate analysis of Y168F AANAT revealed a distinct change in the higher pH values of the pH-rate profile found on the log(kcat/Km) versus pH plot (Fig. 4B). Although the acidic limb with a pKa of 7.3 was maintained, the basic limb of the wild-type profile was now absent, suggesting that Tyr-168 has a pKa of 8.5 and that Tyr-168 should be neutral for optimal activity.

To further probe the active site structure of Y168F AANAT, the susceptibility of Y168F AANAT acetyltransferase activity to inhibition by the bisubstrate analog inhibitor (compound 2) was examined. Using a Dixon analysis, the extrapolated K for 50 mM was found to be essentially identical to that of compound 2 inhibition of the wild-type AANAT enzyme, arguing for the absence of large structural perturbation.

Alkyltransferase Properties of Y168F—The steady-state kinetic parameters for the alkyltransferase activity (9, 10, 12) of Y168F AANAT with N-bromoacetyltryptamine (compound 1) were measured and are displayed in Table II. They reveal a 290-fold reduction in kcat for the Y168F mutant enzyme compared with wild-type AANAT. The Km values for the substrates of this reaction indicate that the apparent affinity of both substrates for the enzyme increased. The Km for CoASH dropped 5-fold (comparable to the Km drop for acetyl-CoA in the acetyltransferase reaction), whereas that for N-bromoacetyltryptamine (compound 1) was reduced more dramatically by 120-fold. Because the chemical step is likely to be rate-determining for this mutant (10), we can make the assumption that rapid equilibrium conditions apply. Under these conditions, the measured substrate Km is representative of the dissociation constant (K). The fact that both the wild-type enzyme and mutant yielded similar K values for the bisubstrate analog (compound 2) indicates that the rate of product release for both enzymes is similar. Therefore, the reduced K for N-bromoacetyltryptamine (compound 1) is suggestive of an increased affinity of this substrate in this case.

X-ray Structure of Y168F AANAT—The structural consequences of changing Tyr-168 to Phe were examined further via x-ray crystallography. The co-crystal structure of Y168F AANAT...
bound to the bisubstrate analog inhibitor (compound 2) was determined at 2.3-Å resolution (see “Experimental Procedures” and Table III for a full account of the structure determination and refinement). The mutant enzyme itself is virtually identical to the wild-type enzyme bound to compound 2 (root mean square difference (r.m.s.d.) = 0.17 Å for 166 α-carbon pairs, which is within the error of the method at this resolution limit), and residue 168 showed no significant structural changes (backbone atom r.m.s.d. = 0.06 Å, maximal difference in side chain atomic position = 0.15 Å). There is no experimental evidence and insufficient space for a water molecule replacing the vacancy of the wild-type Tyr-168 hydroxy group. The mode of inhibitor binding is, however, markedly different. Instead of showing two binding conformations (cis (major) and trans (minor)) for the inhibitor within the AANAT active site, the x-ray structure of Y168F AANAT bound to compound 2 shows only the cis-conformation (r.m.s.d. between the two cis-conformations = 0.5 Å) (Fig. 5). The r.m.s.d. for the tryptamine moiety between the current structure and the wild-type enzyme is 1.2 Å, suggesting that the tryptamine makes similar but measurably different interactions with the enzyme active site.

**Studies on H120Q and H122Q AANAT Mutants**—The proposed role for one or both of these histidine residues is that of a general catalytic base, possibly via a proton wire of water molecules (11). We proceeded to convert each residue to glutamine singly and together to determine their potential roles as a general base. Values for the steady-state kinetic parameters of the acetyl transfer reactions catalyzed by the H120Q and H122Q single mutants as well as the H120Q/H122Q double mutant are given in Table I. For the H120Q and H122Q single mutants, the $k_{cat}$ values are nearly identical, and the $K_m$ values for acetyl-CoA changed by only a factor of 2–3-fold. The largest changes can be seen in the $K_m$ values found for tryptamine; 1.22 mM for H120Q and 4.78 mM for H122Q, representing increases by 7- and 28-fold, respectively. These results are in good agreement with the results determined by Hickman et al. (11). To further understand these changes, viscosity and pH studies were carried out (Figs. 4 and 6 and Table IV). The viscosity experiments revealed that, whereas H120Q-catalyzed acetyl transfer was partially limited by product release/conformational change (slope effect = +0.40), H122Q was now predominantly limited by a non-diffusional step (slope effect = +0.13). Thus, the small $k_{cat}$ effect for H122Q (30% drop) likely represents a 6-fold loss in the rate of the chemical step, as derived from Equation 1. Furthermore, pH-rate studies on H122Q acetyltransferase activity showed the near disappearance of the acidic limb ($pK_a$ 7.3) of the pH-rate profile (Fig. 4C), whereas the profile of H120Q was virtually unchanged (data not shown). It is therefore reasonable to assign the wild-type AANAT ionizable group with $pK_a$ 7.3 to His-122.

The fact that the substitution of each of these residues individually had relatively small effects on the $k_{cat}$ of AANAT and that the two histidine residues are very close in space within the structure of the enzyme, it was hypothesized that they might play redundant catalytic roles. To explore this possibility, we prepared the double mutant H120Q/H122Q AANAT. The acetyltransferase $k_{cat}$ of H120Q/H122Q AANAT is 3.1 s$^{-1}$ (8-fold drop compared with the wild-type enzyme), which is significantly diminished in contrast to the minimal $k_{cat}$ effects observed with either of the single mutant proteins. The small viscosity effect of this mutant (slope = +0.14) implies that the chemical step is rate-determining and that there is an ~40-fold drop in the rate of the chemical step of H120Q/H122Q AANAT compared with wild-type AANAT. These results suggest that there is functional overlap between His-120 and His-122.

**Studies on H120A and H122A AANAT Mutants**—The His-
The double mutant showed a striking drop in the wild-type enzyme, which is 270-fold lower than the value of 25 s⁻¹ found in other GNAT family members.

Alanine double mutant was the change in the wild-type AANAT. Another significant effect observed in the AANAT mutants to gain further insight into AANAT mechanism. Unlike previously described AANAT mutants that express protein at amounts comparable to wild-type AANAT, which is more likely to be a hydrogen bond acceptor than hydrogen bond donor at pH 7. Based on the site-directed mutagenesis studies described here, we can now reasonably assign the residues responsible for the basic, acidic, and reactive ester by enzymes constitutes a critical step in protein synthesis, antibiotic detoxification, chromatin remodeling, melatonin biosynthesis, and a variety of other important biochemical processes. Understanding the strategies used by the various enzymes responsible for this transformation could have important implications for functional genomics and drug design.

**Roles of Catalytic Residues in Serotonin N-Acetyltransferase**

**TABLE II**

|                     | Wild type a | Y168F b | Wild type/ Y168F |
|---------------------|-------------|---------|-----------------|
| $k_{cat}$ (s⁻¹)     | 2.7         | 0.009±0.001 | 290             |
| CoASH $K_m$ (mM)    | 0.15        | 0.03±0.008 | 5               |
| N-Bromoacetyltryptamine | 3.6       | 0.03±0.01 | 120             |

a Taken from Ref. 9.

b Performed as described under “Experimental Procedures.”

**TABLE III**

| Statistics from the crystallographic analysis of Y168F bound to compound 2 |
|--------------------------------------------------|
| **Intenstity data processing**                    |
| Resolution                                       | 20 to 2.3 A |
| $R_{sym}$                                        | 3.7% (9.2%) a |
| No. of measurements                              | 75,910      |
| No. of unique reflections                        | 7188        |
| Completeness                                     | 91.9% (93.0%) a |
| $<i/oI>$                                         | 25.2 (14.1) |
| **Refinement statistics**                        |
| Resolution                                       | 20 to 2.3 A |
| Completeness                                     | 91.9%       |
| $R_{cryst}/R_{free}$                             | 19.3/24.6%  |
| $<B>$ for Y168F AANAT/2                          | 22/33 A²    |
| r.m.s.d. bond length                             | 0.007 Å     |
| r.m.s.d. bond angle                              | 1.3 °       |
| Cross-validated Luzzati coordinate error²        | 0.34 Å      |
| Cross-validated sigmaa coordinate error²         | 0.31 Å      |

a Statistics in parentheses denote high resolution shell data.

b The cross-validated Luzzati and sigmaa coordinate errors for wild-type AANAT are 0.40 and 0.34 Å, respectively.

**DISCUSSION**

Catalysis of amide bond formation between a primary amine and reactive ester by enzymes constitutes a critical step in protein synthesis, antibiotic detoxification, chromatin remodeling, melatonin biosynthesis, and a variety of other important biochemical processes. Understanding the strategies used by the various enzymes responsible for this transformation could have important implications for functional genomics and drug design. The GNAT superfamily shows a conserved structure, particularly regarding acetyl-CoA binding, as well as an apparent conservation in sequential kinetic mechanism. However, it is increasingly apparent that key catalytic residues in these enzymes are not conserved structurally and functionally. One of the reasons for this finding may be the apparent chemical ease of the ester aminolysis reaction (20). Compared with cleavage of an amide bond, the spontaneous rate of ester hydrolysis is considerably faster. GNAT family members lack a structurally obvious oxyanion hole (21) and appear not to commit large binding energy to tetrahedral intermediate stabilization (12).

Other modes of potential catalytic enhancement of the reaction rate involving acid-base catalysis are clearly plausible. Indeed, the previously reported pH-rate profile of AANAT showed the importance of deprotonation of one enzymatic group with a pH of 7.3 (acidic limb) and protonation of a second enzymatic group with a pH of 8.5 (basic limb) (7). Based on the site-directed mutagenesis studies described here, we can now reasonably assign the residues responsible for the acidic limb to His-122 and the basic limb to Tyr-168. Thus, the protonated imidazole (imidazolium) of His-122 and/or the deprotonated phenol (phenolate) of Tyr-168 appears to render the enzyme inactive.

One reason why the imidazolium would fail to function catalytically would be if the imidazolium were necessary to serve as a general base. However, the H122Q mutation resulted in only a 6-fold drop in the chemical step, a reduction well below the maximal effect of general base residue contributions in other enzyme reactions, where 10,000-fold rate effects upon deletion of general base residues are not uncommon (22, 23). It was a formal possibility that the nearby residue His-120 could function as the catalytic base, “rescuing” the phenotype of the mutant. Indeed, His-to-Gln double mutations resulted in a 40-fold drop in the chemical step. A 40-fold drop in the rate of the chemical step might well be sufficient to account for the contribution of a general base to this reaction (24, 25). For example, if the pH-rate profile of the substrate ammonium group is 8.8 in the active site (lowered 1.2 units from the value in free solution), then the maximal acceleration that a general base could be expected to produce at pH 7 (to generate the neutral amine) is 60-fold. This calculation comes from the fact that the pH-rate profile of the neutral amine form at equilibrium at pH 7, and this form would not require base catalysis. Thus, the proton wire model proposed by Hickman et al. (11) is consistent with these data.

Although a catalytic base function is certainly possible, the His-120 and His-122 residues may be accomplishing more than facilitation of deprotonation because the His-to-Ala double mutations were so deleterious. The very large effect on the $k_{cat}/K_m$ for this mutant compared with the His-to-Gln double mutant implies an important role for these residues in hydrogen bonding, indirectly organizing the AANAT active site. It is likely that the acid limb of the wild-type enzyme is due to the His-122 imidazolium ion disrupting this hydrogen bonding/active site organizing function. In this regard, it is proposed that His-122 is more likely to be a hydrogen bond acceptor than hydrogen bond donor at pH 7. Based on the site-directed mutagenesis studies described here, we can now reasonably assign the residues responsible for the acidic limb to His-122 and the basic limb to Tyr-168. Thus, the protonated imidazole (imidazolium) of His-122 and/or the deprotonated phenol (phenolate) of Tyr-168 appears to render the enzyme inactive.

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bond donor and that the imidazolium ion could not subserve this hydrogen bond acceptor function.

In contrast to the relatively modest importance ascribed to general base function in AANAT, another member of the GNAT family, GCN-5 (as well as its close relative PCAF), has been shown to have a critical glutamate serving as a general base (26), predicted to be functionally similar to AANAT His-120 based on sequence (and three-dimensional) alignment between GCN-5 and AANAT (27). Mutation of this GCN-5 Glu residue to Gln results in a 1000-fold acetyltransferase rate drop (28). It is possible that a greater impact of a catalytic base for this enzyme could be needed if the protein substrate lysine has a higher pK_a when bound to the enzyme compared with that of tryptamine in AANAT. Alternatively, there could be subtle structural effects that are independent of base function.

In contrast to the His residues examined, which are relatively far from the bisubstrate analog in the x-ray crystal structure, Tyr-168 appears to make a direct hydrogen bond to the sulfur atom of the bisubstrate analog (compound 2). The GNAT sequence alignment of Neuwald and Landsman (3) shows a tyrosine residue present at this position in several other related enzymes such as spermidine N-acetyltransferase and some of the aminoglycoside N-acetyltransferases. However, other enzymes within the family, including several histone acetyltransferases, do not have a conserved tyrosine at this position, but have a phenylalanine in its place. Confirming previous work (11), mutation of Tyr-168 to Phe led to a significant k_cat reduction in AANAT (30-fold). The use of viscosity studies in the previous (7) and present work allowed us to quantitate the isolated effect (150-fold reduction) on the chemical step. This is consistent with either a general acid function or substrate-orienting role. It should be noted that not only could hydrogen bonding to this sulfur enhance the leaving group capacity of the CoA moiety, it can render the attacked carbonyl more electrophilic by an inductive effect. Both of these effects could enhance the chemical reaction rate. The relatively low pK_a of this tyrosine (8.5) gleaned from the pH rate suggests that the more acidic character of this residue could enhance proton transfer to the sulfur. How the enzyme reduces the pK_a of Tyr-168 (from 10, the value of free tyrosine, to 8.5) is not fully understood. A hydrophobic active site would tend to raise its pK_a by destabilizing charge build-up. Perhaps strategies proposed for pK_a lowering of Tyr in other systems (29, 30) are involved.

It was rather unexpected that replacement of Tyr-168 with Phe resulted in a 10-fold drop in the K_m for acetyl-CoA. Based on calorimetry studies carried out by Dyda and co-workers (31), this finding probably represents an enhanced affinity of the
The tryptamine pocket. However, the substrate. Further support of this idea is the finding that product release of the Y168F AANAT mutant was slowed by ~30-fold compared with the wild-type protein based on the viscosity studies. Moreover, as described above, CoASH was found to have a 5-fold lower \( k_{cat} \) in the alkyltransferase assay catalyzed by Y168F AANAT. It is likely that the presumed higher affinity of CoASH for the mutant enzyme contributes to the slowing of its off-rate. This seemed unusual because the loss of a hydrogen bond between the CoA sulfur and Tyr-168 leads to a gain of affinity. A gross structural perturbation that might account for this effect was ruled out by obtaining the x-ray structure of the complex between the bisubstrate analog (compound 2) and the mutant. The relative free energy change of forming the hydrogen bond between Tyr-168 and the CoA sulfur versus deleting a hydroxyl group from a hydrophobic environment may favor the latter. A related effect was observed in the enzyme ketosteroid isomerase, where mutation of a hydrogen-bonding tyrosine (Tyr-14) led to an increase in ground-state binding as well as a decrease in \( k_{cat} \) (32). Noting that this class of mutation is relatively rare in enzymology, Warshel (33) has described this model as “ground-state destabilization” in which a wild-type enzyme residue acts in part by raising the free energy of the ground state to lower the relative barrier to the transition state.

It is clear that many of the mutants showed elevations of the \( K_m \) for tryptamine in the acetyltransferase reaction. This may seem surprising because the His and Tyr residues are far from the tryptamine pocket. However, the \( K_f \) (1 mM) for tryptamine is ~6-fold higher than the \( K_m \) (0.17 mM) with the wild-type enzyme (31), as would be expected when product release of CoASH is rate-determining (34). Thus, the \( K_m \) values in the millimolar range in the catalytically defective mutants will result in part from a change in the ratio of the chemical and product release steps.

The alkyltransferase activity catalyzed by AANAT, relevant for drug design, is an unusual and incompletely understood aspect of its molecular recognition. It is notable that Tyr-168 plays a large role in the chemical step (10), with mutation to Phe resulting in a 290-fold rate drop. One can surmise an important orientational role or even a catalytic base function in this residue. Consistent with the acetyltransferase reaction, there was a 5-fold drop in the \( K_m \) for CoASH, suggestive of the higher affinity. Quite strikingly, the \( K_m \) for N-bromacetyltryptamine (compound 1) dropped ~120-fold in the Y168F AANAT mutant compared with the wild-type enzyme. This was unexpected not only because of the distance between the tyrosine and this moiety observed in the x-ray structure of the wild-type bisubstrate analog complex, but also because the x-ray structure of the mutant showed little change compared with the wild-type enzyme. The x-ray structure did show, however, that the mutant displayed only one conformation of the bisubstrate analog (compound 2) as opposed to the two conformations seen in the wild-type complex. This conformation is most similar (but not identical to) the cis-conformation of the wild-type complex. Given these findings, it is plausible that the cis-conformation corresponds to the acetyltransferase reaction and the trans-conformation to the acetyltransferase reaction. Consistent with this possibility, the tryptamine \( K_m \) (2.3 mM) for the Y168F acetyltransferase reaction is 14-fold elevated compared with the wild-type system, a larger increase than would be predicted based on relative chemical step rate constant decrease and suggestive of decreased ground-state binding affinity for this reaction conformation. The especially enhanced apparent affinity of compound 1 for Y168F AANAT may be related in part to the modified contacts observed compared with the cis-inhibitor conformation in the wild-type complex. In future mutagenesis and kinetic studies, the accuracy of this model can be investigated by altering residues that seem to make more important interactions with the cis- rather than the trans-conformation of compound 2 bound to AANAT.

**CONCLUSION**

In this report, we explored the kinetic contributions of His-122, His-124, and Tyr-168 to AANAT catalytic action. We were able to convincingly assign the \( pK_a \) values of His-122 and Tyr-168, which are likely to be responsible for the acidic and basic limbs of the pH-rate profile of the wild-type enzyme, and discussed how they might contribute to catalysis. We demonstrated the paradox that the loss of a hydrogen bond between the CoA analog (compound 2) and Tyr-168 of the enzyme leads to an increase in apparent affinity for CoA. We showed that mutation of Tyr-168 leads to a marked enhancement of substrate binding in the acetyltransferase reaction catalyzed by AANAT and were able to correlate this with an x-ray crystallographic structural appearance of only a single bound conformation for compound 2. These studies provide new insights into catalysis by the GNAT superfamily and suggest novel opportunities for the design of potent and selective AANAT inhibitors.

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