

Indoleamine Analogs as Probes of the Substrate Selectivity and Catalytic Mechanism of Serotonin *N*-Acetyltransferase*

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Serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase (AANAT)) catalyzes the reaction of serotonin (or tryptamine) with acetyl-CoA to form *N*-acetylserotonin (or *N*-acetyltryptamine) and is responsible for the melatonin circadian rhythm in vertebrates. This study evaluates a series of indoleamine analogs as alternate substrates of AANAT. 3-Indolepropylamine and 3-indolebutylamine were chemically synthesized and found to be processed by AANAT, although 20- and 60-fold less efficiently compared with the natural substrate serotonin, respectively. Racemic α -methyltryptamine and *N*^ω-methyltryptamine were also shown to be substrates for AANAT, again with reduced k_{cat} and k_{cat}/K_m compared with serotonin. The enzyme did exhibit ~9:1 stereoselectivity for the *R*-enantiomer of α -methyltryptamine versus the *S*-enantiomer. By measuring the enzymatic rates versus increasing buffer microviscosity, it was demonstrated that diffusional release of product is most likely the principal rate-determining step for the enzymatic transformation of tryptamine (which has similar k_{cat} and k_{cat}/K_m compared with serotonin). Analysis of k_{cat} and k_{cat}/K_m versus pH for the poor substrate *N*^ω-methyltryptamine showed that an ionizable group on the enzyme with $pK_a \sim 7$, required to be in its deprotonated form, may be important in catalysis. The α -methyltryptamine analog α -trifluoromethyltryptamine was not processed by the enzyme, but served as a modest competitive inhibitor. Taken together with the pH-rate analysis, these results favor a model in which the serotonin substrate binds to the enzyme as the positively charged ammonium salt, and nucleophilicity of the amine is important in enzyme-catalyzed acetyl transfer.

Serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase (AANAT)¹) is the enzyme responsible for the circadian rhythmic cycle of melatonin (5-methoxy-*N*-acetyltryptamine) (1). AANAT catalyzes the conversion of serotonin to *N*-acetylserotonin, the direct precursor to melatonin (Fig. 1). AANAT is expressed primarily in the pineal glands of vertebrates, where acetyltransferase activity rises in the night and falls in the daytime, regulating the production of melatonin. The recent cloning of AANAT (1) has facilitated advances in the

understanding of its regulation (2) and enzyme mechanism (3). In principle, a detailed understanding of the substrate selectivity and catalytic mechanism of AANAT could lead to the development of specific AANAT inhibitors that might have therapeutic value in sleep and mood disorders.

AANAT is a 24-kDa protein that belongs to the motif A/B superfamily, which includes >150 proteins, many of which are believed to be acetyltransferase enzymes (1, 3). This superfamily is defined by the presence of two weakly conserved tandem sequences of ~15 amino acids called motifs A and B. AANAT is itself highly conserved among vertebrates, displaying ~80% amino acid identity among sheep, chicken, rat, and human AANATs (1). Recently, recombinant sheep AANAT was overproduced and purified from *Escherichia coli* (3). Its kinetic mechanism was shown to be sequential (ternary complex) Ordered Bi Bi, with acetyl-CoA binding before serotonin (or the alternative substrate tryptamine) (3).

Previous studies of the substrate specificity of AANAT have revealed that the indole function is particularly important for efficient turnover (4). The design of specific AANAT inhibitors will be augmented by a thorough analysis of the tolerance of the AANAT active site for alternate substrates. The processing of alternative substrates can also yield insights into the catalytic and kinetic mechanisms of the enzyme. As part of a previous investigation of AANAT, a simple kinetic acetyltransferase assay was reported in which free CoASH was indirectly monitored by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (3). In principle, this assay allows a variety of serotonin analogs to be tested as AANAT substrates in a convenient way. In this study, we report the results of the effects of substrate alterations on AANAT processing.

EXPERIMENTAL PROCEDURES

Materials—Tryptamine, racemic α -methyltryptamine, *N*^ω-methyltryptamine, 5,5'-dithiobis(2-nitrobenzoic acid), Mops, Mes, and Epps were purchased from Sigma. 3-Bromo-1,1,1-trifluoroacetone, *O*-benzylhydroxylamine, 3-indolepropionic acid, 3-indolebutyric acid, LiAlH₄, dimethylformamide, and tetrahydrofuran (anhydrous) were purchased from Aldrich. Sucrose was purchased from Bio-Rad. Recombinant glutathione *S*-transferase-AANAT was overexpressed and purified as described previously (3). All reagents and solvents were used as purchased without further purification. Preparatory TLC was performed on 1000- μ m silica gel plates (20 × 20 cm; Analtech Uniplate®). Silica gel (200–425 mesh) for column chromatography was purchased from Fisher.

Instruments—¹H, ¹³C, and ¹⁹F NMR experiments were performed on a Bruker 400-MHz spectrometer. Optical rotations were measured using a Jasco DIP-370 digital polarimeter.

3-Indolepropionyl-*O*-benzylhydroxamate (5)—To a solution of 3-indolepropionic acid (**3**; 2.0 g, 10.6 mmol) in dimethylformamide (100 ml) was added Et₃N (4.4 ml, 31.8 mmol) and *O*-benzylhydroxylamine hydrochloride (3.4 g, 21.1 mmol). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (4.0 g, 21.1 mmol) was added to the solution at 0 °C, and the resulting mixture was allowed to warm up to room temperature. After 48 h of stirring under an atmosphere of N₂, undissolved material was removed from the reaction mixture by filtration, and the filtrate was concentrated *in vacuo* to remove dimethylform-

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¹ The abbreviations used are: AANAT, arylalkylamine *N*-acetyltransferase (serotonin *N*-acetyltransferase); HRMS, high resolution mass spectrometry; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Epps, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid; EtOAc, ethyl acetate.

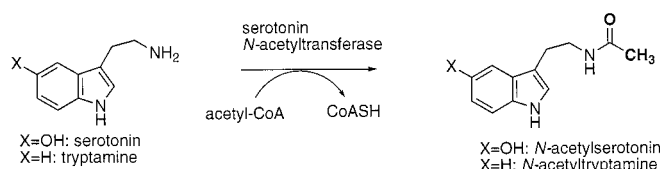


Fig. 1. Serotonin *N*-acetyltransferase-catalyzed conversion of serotonin to *N*-acetylserotonin.

amide. The resulting residue was partitioned between 0.5 N HCl (100 ml) and EtOAc (200 ml). The organic layer was washed with 100 ml of 1 N NaHCO₃ and brine; dried over MgSO₄; and concentrated *in vacuo* to give fairly pure product as a white solid (1.8 g, 58%), which was recrystallized from hot MeOH to give 1.2 g (first crop) of pure compound **5**. TLC $R_F = 0.24$ (EtOAc/hexanes, 1:1). ¹H NMR (400 MHz, Me₂SO-*d*₆) δ 2.33 (t, $J = 7.6$ Hz, 2 H), 2.94 (t, $J = 7.6$ Hz, 2 H), 4.74 (s, 2 H), 6.98 (t, $J = 7.3$ Hz, 1 H), 7.05–7.10 (m, 2 H), 7.28–7.41 (m, 6 H), 7.53 (d, $J = 7.8$ Hz, 1 H), and 10.78 (br s, 1 H); ¹³C NMR (100 MHz, Me₂SO-*d*₆) δ 22.97, 35.57, 79.04, 113.61, 115.67, 120.46, 120.62, 123.20, 124.57, 129.27, 130.48, 130.56, 131.13, 138.38, 138.54, and 171.38. HRMS calculated for C₁₈H₁₈N₂O₂ + H⁺: 295.1448; found: 295.1448.

3-Indolebutyryl-*O*-benzylhydroxamate (6)—Following the procedure described above for the preparation of compound **5**, reaction of 3-indolebutyric acid (**4**; 0.8 g, 4.1 mmol), *O*-benzylhydroxylamine hydrochloride (1.3 g, 8.1 mmol), Et₃N (1.7 ml, 12.3 mmol), and 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (1.5 g, 8.1 mmol) in dimethylformamide (75 ml) for 3 days afforded the crude product as a tan oil (1.0 g, 77%). Purification of this material on silica gel using a mixture of EtOAc/hexanes (1:1) as the eluent afforded 0.7 g (54%) of pure compound **6** as a colorless oil. TLC $R_F = 0.27$ (EtOAc/hexanes, 1:1). ¹H NMR (400 MHz, CDCl₃) δ 1.92 (m, 4 H), 2.65 (m, 2 H), 4.77 (s, 2 H), 6.75 (br s, 1 H), 7.00 (t, $J = 7.4$ Hz, 1 H), 7.08 (t, $J = 7.4$ Hz, 1 H), 7.22–7.26 (m, 6 H), 7.45 (d, $J = 7.8$ Hz, 1 H), and 8.12 (br s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 26.84, 28.14, 35.01, 80.65, 113.82, 117.47, 121.33, 121.64, 124.40, 128.42, 129.84, 131.18, 131.32, 131.79, 137.77, 138.82, and 173.70.

HRMS calculated for C₁₉H₂₀N₂O₂ + H⁺: 309.1605; found: 309.1612

3-Indolepropylamine (1) (5)—To a solution of compound **5** (400 mg, 1.4 mmol) in dry tetrahydrofuran (25 ml) was added LiAlH₄ (4.1 ml, 1.0 M in tetrahydrofuran), and the resulting mixture was refluxed under N₂. After 3 days of refluxing, excess LiAlH₄ was quenched by slow addition of 0.5 N HCl (5 ml). The mixture was filtered, and the filtrate was diluted with EtOAc (50 ml) and 0.5 N HCl (25 ml). The aqueous layer was basified with 1 N NaOH to pH >10 and then extracted with EtOAc (2 × 50 ml). The organic layers were combined, dried over MgSO₄, and evaporated *in vacuo* to give 38 mg of compound **1** (yield based on recovered compound **5**, 90%), which appeared as a single spot on TLC. $R_F = 0.25$ (CH₂Cl₂/MeOH/NH₄OH, 100:10:2). For analytical purposes and for use in the enzyme assay, the material was further purified using preparative TLC to give compound **1** as a white solid, which was quantitatively converted to its hydrochloride salt and stored at –20 °C until needed. ¹H NMR (400 MHz, CDCl₃) δ 1.56 (br s, 2 H), 1.76–1.84 (m, 2 H), 2.69–2.75 (m, 4 H), 6.89 (s, 1 H), 7.04 (t, $J = 7.5$ Hz, 1 H), 7.12 (t, $J = 7.5$ Hz, 1 H), 7.27 (d, $J = 8.1$ Hz, 1 H), 7.53 (d, $J = 7.9$ Hz, 1 H), and 8.11 (br s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 22.65, 34.14, 42.18, 111.29, 116.34, 119.06, 119.26, 121.37, 122.05, 127.65, and 136.54. HRMS calculated for C₁₁H₁₄N₂ + H⁺: 175.1236; found: 175.1237.

3-Indolebutylamine (2) (5)—Following the procedure described above for the preparation of compound **1**, 3-indolebutyryl-*O*-benzylhydroxamate (**6**) (160 mg, 0.54 mmol) was reacted with LiAlH₄ (7.4 ml, 1.0 M in tetrahydrofuran) to give 7 mg of compound **2** (yield based on recovered compound **6**, 80%). TLC $R_F = 0.21$ (CH₂Cl₂/MeOH/NH₄OH, 100:10:2). ¹H NMR (2-HCl, 400 MHz, D₂O) δ 1.51–1.67 (m, 4 H), 2.69 (t, $J = 7.0$ Hz, 2 H), 2.85 (t, $J = 7.4$ Hz, 2 H), 7.01 (t, $J = 7.9$ Hz, 1 H), 7.07 (s, 1 H), 7.10 (t, $J = 7.1$ Hz, 1 H), 7.36 (d, $J = 8.1$ Hz, 1 H), and 7.55 (d, $J = 8.0$ Hz, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 25.19, 27.63, 33.87, 42.32, 111.28, 116.82, 119.12, 119.24, 121.37, 122.03, 127.65, and 136.46. HRMS calculated for C₁₂H₁₆N₂ + H⁺: 189.1393; found: 189.1396.

(±)- α -Trifluoromethyltryptamine (9) (6)—Compound **9** was synthesized using the vinylnitroso cycloaddition strategy of Zimmer and Reissig (**6**) and gave ¹H NMR and mass spectra consistent with those reported (**6**). Measurement of the pK_a for compound **9** was carried out by pH titration of the ¹H NMR spectrum of compound **9** with 3 mM compound **9** in 20% D₃COD/D₂O with 200 mM NaCl at 25 °C. The pH was adjusted with NaOD and DCl, and the chemical shifts of the

methyl proton (α to the amine function and the trifluoromethyl group) were standardized *versus* trimethylsilylpropionate. The data were fit to a modified Hill equation (7) that fit smoothly to a one-proton transfer equilibrium with a calculated pK_a of 5.4 ± 0.1.

(±)-*N*-Acetyl- α -methyltryptamine—A solution of α -methyltryptamine (53 mg, 0.3 mmol) in CH₂Cl₂ (10 ml) was treated with acetic anhydride (30 μ l, 0.32 mmol) and Et₃N (37 μ l, 0.5 mmol). The reaction flask was wrapped with aluminum foil, and the mixture was stirred at room temperature under N₂. Reaction progress was monitored by TLC, and the reaction was essentially complete after 1 h. The reaction mixture was diluted with CH₂Cl₂ to ~20 ml and successively washed with 0.5 N HCl (20 ml), 1 N NaHCO₃ (15 ml), H₂O (15 ml), and brine. The organic layer was dried (MgSO₄) and then evaporated *in vacuo* to give the product as a clear oil (70 mg, 108% mass balance). ¹H and ¹³C NMR analyses showed that excess mass was due to a minor contaminant of acetic anhydride. A sample for analysis was purified using preparative TLC. TLC $R_F = 0.49$ (CH₂Cl₂/MeOH, 10:1). ¹H NMR (400 MHz, CDCl₃) δ 1.07 (d, $J = 6.6$ Hz, 3 H), 1.81 (s, 3 H), 2.84 (dd, $J = 5.9$ and 1.8 Hz, 2 H), 4.22–4.36 (m, 1 H), 5.67 (br d, $J = 7.8$ Hz, 1 H), 6.90 (s, 1 H), 7.02 (t, $J = 7.9$ Hz, 1 H), 7.09 (t, $J = 7.9$ Hz, 1 H), 7.27 (d, $J = 8.0$ Hz, 1 H), 7.53 (d, $J = 7.9$ Hz, 1 H), and 8.52 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 19.62, 22.77, 31.07, 45.16, 110.66, 110.99, 118.22, 118.70, 121.24, 122.26, 127.33, 135.65, and 169.17. HRMS calculated for C₁₃H₁₆N₂O⁺: 216.1264; found: 216.1260.

***N*-Acetyl-*N*^ω-methyltryptamine**—Following the procedure described above for the preparation of *N*-acetyl- α -methyltryptamine, *N*^ω-methyltryptamine (78 mg, 0.45 mmol) in CH₂Cl₂ (10 ml) and Et₃N (125 μ l, 0.9 mmol) was acetylated with acetic anhydride (85 μ l, 0.9 mmol) to give the *N*-acetyl-*N*^ω-methyltryptamine product as a white solid (100 mg, 100%). *cis*- and *trans*-rotamers were observed in ¹H and ¹³C NMR spectra about the N–C=O bond at 298 K at a ratio of ~55:45. ¹H NMR (400 MHz, CDCl₃) δ 1.88 and 2.13 (2 s, 1.6 H and 1.4 H), 2.86–2.98 (m, 5 H), 3.53 and 3.63 (2 t, $J = 6.5$ and 6.6 Hz, 1.1 H and 0.9 H), 6.92 and 6.98 (2 s, 0.55 H and 0.45 H), 7.06–7.15 (m, 2 H), 7.31 (t, $J = 8.3$ Hz, 1 H), 7.50 (d, $J = 7.6$ Hz, 0.6 H), 7.58 (d, $J = 7.5$ Hz, 0.4 H), and 8.29 and 8.41 (2 br s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 21.84, 22.84 (COCH₃), 24.16, 25.12 (NCH₃), 34.39, 37.79 (NCH₂CH₂), 49.69, 52.37 (NCH₂CH₂), 112.16, 112.45, 112.80, 114.02, 119.05, 119.61, 120.25, 120.52, 122.90, 122.95, 123.16, 123.30, 126.83, 127.95, 137.26, 138.26 (indole), 171.70, and 171.99 (C=O). HRMS calculated for C₁₃H₁₆N₂O⁺: 216.1264; found: 216.1263.

Acetyltransferase Kinetic Assays of Amine Analogs—Preparation of purified recombinant sheep glutathione *S*-transferase-AANAT has been described, and it has shown to be nearly identical in its kinetic behavior to purified glutathione *S*-transferase-free AANAT (**3**). Assays of the amine analog substrates were performed essentially as previously reported using a spectrophotometric assay in which CoASH levels were monitored indirectly by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (**3**). To verify that *N*^ω-methyltryptamine was forming the acetylated product, a large-scale reaction (20 mM, 3 ml) was performed, and *N*^ω-acetyl-*N*^ω-methyltryptamine was isolated and shown to have identical ¹H NMR and TLC properties compared with the authentic material. For K_m measurements, at least five amine substrate concentrations ranging from <0.3 K_m to >3 K_m were employed in the presence of near-saturating concentrations of acetyl-CoA (>3 K_m). Rates were measured under initial conditions (<10% turnover of the limiting substrate). All assays were performed in duplicate, and rate values generally agreed within 10%. Kinetic constants were calculated by fitting the data to the Michaelis-Menten equation using a nonlinear curve fit (Kaleidograph), and the values are shown as means ± S.E. in Table I.

α -Trifluoromethyltryptamine (9) Inhibition Experiment—After showing that α -trifluoromethyltryptamine was not processed by AANAT (k_{cat}/K_m at least 1000-fold lower than for compound **7**), it was tested as a competitive inhibitor using a Dixon analysis *versus* the substrate *N*^ω-methyltryptamine. A range of five concentrations of compound **9** (up to 10 mM) was used in the inhibition experiment. The assay conditions were similar to those described previously (**3**) with minor modifications. MeOH (final concentration of 5%) was included in the assay to improve the solubility of compound **9**. Controls demonstrated that this concentration of MeOH had no detectable effect on turnover rate. In addition, 0.1 M Mes (pH 6.1) with 0.1 M NaCl was used in place of the standard phosphate buffer (**3**). Two separate assays were performed at two fixed subsaturating concentrations (4 and 6 mM) of *N*^ω-methyltryptamine ($K_m = 4$ mM). The concentration of acetyl-CoA ($K_m = 0.4$ mM) was kept constant and near-saturating (2 mM). Dixon plots were fit using linear regression. The K_i was calculated assuming a competitive inhibition model (*versus* *N*^ω-methyltryptamine) from the slope of the line using the steady-state kinetic equation (Equation 1).

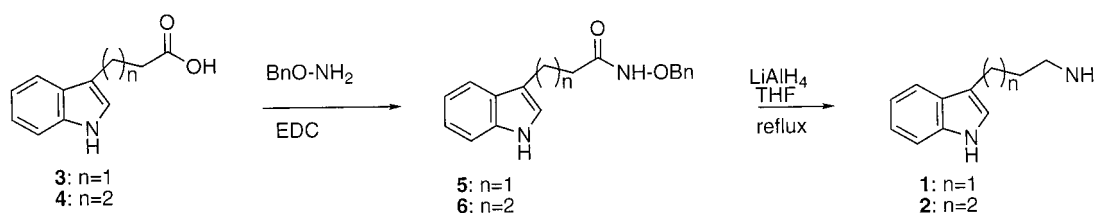


FIG. 2. Synthesis of homologated tryptamine analogs. EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; THF, tetrahydrofuran.

$$\text{Slope} = K_m/V_m K_i[S] \quad (\text{Eq. 1})$$

The calculated K_i values from both experiments were in good agreement, and the values are shown as means \pm S.E. in Table I.

Stereochemical Studies— α -Methyltryptamine (20 mM) and acetyl-CoA (20 mM) were reacted with AANAT (10 μ M) in a 3-ml reaction volume using previously described acetyltransferase conditions (pH 6.8 and sodium phosphate buffer) (3), and reaction progress was monitored with 5,5'-dithiobis(2-nitrobenzoic acid). After 20 min, when the reaction was shown to be \sim 50% complete, the reaction mixture was vortexed vigorously for 2 min. A white precipitate formed, which was removed by centrifugation (3000 \times g, swinging bucket). The resulting solution was diluted with CH_2Cl_2 (10 ml) and H_2O (10 ml). The aqueous layer (pH \sim 7) was separated and extracted with additional CH_2Cl_2 (3 \times 10 ml). The combined organic layers were dried (MgSO_4) and concentrated *in vacuo* to give 6.8 mg (54%) of *N*-acetyl- α -methyltryptamine. The neutral aqueous layer was basified to pH \sim 11 using 1 N NaOH and then extracted with CH_2Cl_2 (3 \times 10 ml). The combined extracts were dried (MgSO_4) and concentrated *in vacuo* to give 5.5 mg (44%) of recovered α -methyltryptamine. The identity and purity of both materials (>95%) were verified by ^1H NMR. The concentrations of the solutions used for measuring optical rotations were verified independently by measuring UV absorption at $\lambda = 279$ nm. The specific rotations are shown under "Results" and gave similar results on two separate occasions. To rule out the possibility that transfer of the acetyl group had occurred non-enzymatically at these concentrations of α -methyltryptamine and acetyl-CoA, the assay mixture, in the absence of AANAT, was stored at 30 $^\circ\text{C}$ for 2 h. No acetyl transfer could be detected (<5%) under these conditions.

Viscosity Studies—All assays were performed with the added viscose sucrose to achieve the desired viscosity. Relative viscosities were previously measured with a Canon-Fenske viscometer at 30 $^\circ\text{C}$ as described previously (8). Sucrose solutions used to achieve the viscosities in this work were 1.0 (0% (w/v) sucrose), 1.55 (16%), 1.99 (23%), 2.50 (30%), and 3.10 (35%). Measurements of k_{cat} and K_m were performed as described previously (3), except that Mops at pH 7.4 and 100 mM NaCl were employed. Under these conditions in the presence of saturating concentrations of *N* $^\omega$ -methyltryptamine or tryptamine, the K_m for acetyl-CoA was found to be 0.3 mM and did not significantly change with added sucrose. The K_m for tryptamine at near-saturating concentrations of acetyl-CoA in the absence of sucrose was found to be 0.12 mM and was <0.1 mM with added sucrose. The K_m for *N* $^\omega$ -methyltryptamine at near-saturating concentrations of acetyl-CoA in the absence of sucrose was found to be 4 mM and fell with increasing sucrose concentrations as described under "Results." The k_{cat} values under these assay conditions without added sucrose were 27 and 2.5 s^{-1} for tryptamine and *N* $^\omega$ -methyltryptamine, respectively.

Rate Versus pH Profile—Using *N* $^\omega$ -methyltryptamine as substrate, the measurements for k_{cat} and K_m were performed in the pH range 6–8.6 following the general spectrophotometric assay procedures previously described (3). Accurate rate measurements were not possible above pH 8.6 because of high background aminolysis/hydrolysis rates of acetyl-CoA. All reactions were initiated with enzyme (which was maintained at pH 6.8, but did not significantly affect the final pH), and the activities proved to be linear for at least 3 min, suggesting that altered kinetics were not caused by protein instability under the assay conditions. Enzyme assays showed Michaelis-Menten kinetic behavior at each pH investigated. All assays were performed with near-saturating concentrations of acetyl-CoA (>3 K_m , verified at each pH) throughout the pH range. The following buffers were used to obtain the pH values described in the experiments: pH 6.1–6.7 (Mes), pH 6.7–7.6 (Mops), and pH 7.7–8.6 (Epps). Fits for the k_{cat} data were performed using Equation 2,

$$\log(k_{\text{cat}})_{\text{app}} = \log(k_{\text{cat}}/(1 + H/K_1)) \quad (\text{Eq. 2})$$

where H is the proton concentration and K_1 is the dissociation constant for the ionizable group that facilitates the reaction in its deprotonated

form. Fits for the k_{cat}/K_m data were performed using Equation 3,

$$\log(k_{\text{cat}}/K_m)_{\text{app}} = \log(k_{\text{cat}}/K_m(1 + H/K_1 + K_2/H)) \quad (\text{Eq. 3})$$

where H is the proton concentration, K_1 is the dissociation constant for the ionizable group that facilitates the reaction in its deprotonated form, and K_2 is the dissociation constant for the ionizable group that facilitates the reaction in its protonated form. Both equations were fit using the computer program Kinetasyst II (Intellikinetics), and the kinetic constants \pm S.E. are shown under "Results."

RESULTS

Homologated Tryptamines—The known substrates serotonin and tryptamine contain an ethyl side chain extending from the indole 3-position. The initial objective of these studies was to determine the tolerance of AANAT for substrate alkyl chain length variations. We thus set out to prepare the alkyl chain-extended propylamine (1) and butylamine (2) analogs of tryptamine (5). Synthesis of these compounds was achieved in two steps, starting from the corresponding commercially available carboxylic acids 3 and 4 (Fig. 2). The carboxylic acids were converted to the hydroxamic acid derivatives 5 and 6 and then exhaustively reduced with LiAlH_4 to afford the desired amines 1 and 2.

Kinetic analysis of these compounds was carried out with recombinant sheep AANAT in the presence of saturating levels of acetyl-CoA and with an indirect spectrophotometric assay that monitors CoASH formation. Both of the chain-extended indoleamine substrates 1 and 2 were found to be AANAT substrates and displayed normal Michaelis-Menten kinetic behavior, although with somewhat reduced catalytic efficiency compared with serotonin (Table I). Propylamine analog 1 was 20-fold reduced in catalytic efficiency, exhibiting both a higher K_m and lower k_{cat} compared with serotonin. Butylamine analog 2 was only 3-fold less active as a substrate compared with propylamine analog 1, and the difference was primarily in K_m . These results show that AANAT has an optimal preference for indole substrates with 3-ethylamine side chains, corresponding to the natural substrate, but is reasonably tolerant of side chain extension by at least two more methylene groups from the indole ring.

Methyl-substituted Tryptamine Analogs—Another important issue related to AANAT selectivity is the ability to accommodate α -branching or nitrogen substitution (secondary amines). Tryptophan (both D- and L-isomers) was shown not to be processed by AANAT. The commercially available racemic α -methyltryptamine (7) and *N* $^\omega$ -methyltryptamine (8) were next evaluated as AANAT substrates (Fig. 3). Both of these analogs were utilized as substrates by AANAT and displayed Michaelis-Menten behavior, although again with diminished catalytic efficiency compared with tryptamine (Table I). In addition to monitoring amine-induced CoASH generation in the presence of AANAT, direct characterization of the acetamide product derivatives of compounds 7 and 8 was accomplished using TLC and ^1H NMR, ruling out indoleamine-promoted acetyl-CoA hydrolysis. Racemic α -methyltryptamine (7) exhibited $k_{\text{cat}} = 16 \pm 0.4 \text{ s}^{-1}$ and $K_m = 2.7 \pm 0.2 \text{ mM}$. *N* $^\omega$ -methyltryptamine (8) was still a less efficient substrate, showing $k_{\text{cat}} = 2.0 \pm 0.1 \text{ s}^{-1}$ and $K_m = 3.8 \pm 0.7 \text{ mM}$. Although the catalytic efficiency (k_{cat}/K_m)

TABLE I
Steady-state kinetic parameters for tryptamine analogs as substrates or inhibitors of AANAT

See "Experimental Procedures" for experimental details.

Tryptamine analog	K_m	k_{cat}	K_i
	mM	s ⁻¹	mM
Serotonin	0.24 ± 0.03	34 ± 1	
Tryptamine	0.17 ± 0.01	25 ± 1	
Compound 1	0.77 ± 0.07	5.5 ± 0.2	
Compound 2	1.7 ± 0.1	4.2 ± 0.1	
(±)- α -Methyltryptamine (7)	2.7 ± 0.2	16 ± 0.4	
<i>N</i> ^ω -Methyltryptamine (8)	3.8 ± 0.7	2.0 ± 0.1	
(±)- α -Trifluoromethyltryptamine (9)			3 ± 1

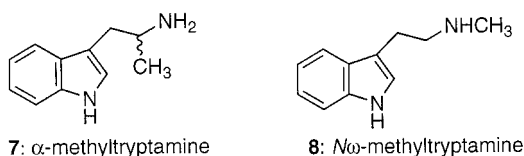


FIG. 3. Methyl-substituted tryptamine analogs.

for compound 8 with AANAT compared with the standard substrate tryptamine was ~300-fold reduced, it was still much greater (>300-fold) than the non-enzyme-catalyzed background acetylation rate. That α -methyltryptamine and *N*^ω-methyltryptamine were AANAT substrates allowed further investigation into active-site stereochemical accessibility and enzymatic rate-determining step(s).

Stereochemical Studies—Most enzyme reactions demonstrate stereoselectivity because of the well defined structure of enzyme active sites. Although racemic α -methyltryptamine (7) was shown to be a substrate for AANAT, the spectrophotometric assay described above was insufficient to reveal potential stereochemical selectivity. A larger scale reaction of AANAT with racemic α -methyltryptamine (7) was allowed to proceed to ~50% completion over 20 min, and the product and the unreacted starting material were isolated in pure form. The specific rotation ($[\alpha]_D^{25}$) of the recovered α -methyltryptamine from the enzyme reaction was found to be $+26^\circ \pm 3^\circ$ ($+32^\circ = 100\%$ *S*-enantiomer (9)), indicating that it was a 9:1 mixture of *S/R*-isomers. Furthermore, the specific rotation of the enzymatic product *N*-acetyltryptamine ($+9^\circ$) confirmed it to be principally the *R*-enantiomer by demonstrating its similar magnitude to and opposite sign from (-8.4°) the chemical acetylation product of the enantiomerically enriched (*S*)- α -methyltryptamine (*i.e.* the recovered starting material from the AANAT reaction). These results establish unequivocally that AANAT shows stereoselectivity for α -substituted tryptamine analogs and, in the case of α -methyl substitution, that the *R*-enantiomer is processed more efficiently than the *S*-enantiomer.

Viscosity Studies—Previous studies have shown that for many enzyme reactions, the rate-determining step can be diffusional release of product rather than the chemical transformation step (10). A particularly effective method for elucidating the nature of the rate-determining step(s) is to examine the effect of increasing viscosity on the kinetics of the reaction (10). By increasing the viscosity of the solution with a microviscogen such as sucrose, rates of diffusional steps are slowed, whereas unimolecular processes are theoretically unaffected. However, large quantities of compounds such as sucrose can cause conformational or other "artificial" effects on enzyme reactions by interacting with the enzyme, substrate(s), or both. To control for these non-diffusional effects, it is helpful to have "poor substrates" for which the rate-determining step can be reason-

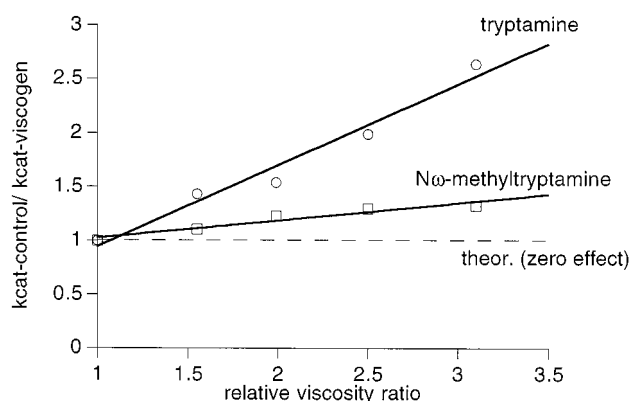


FIG. 4. Plot of $\log(k_{cat}\text{-control}/k_{cat}\text{-viscogen})$ versus relative viscosity ratio for acetyl transfer catalyzed by serotonin *N*-acetyltransferase with tryptamine and *N*^ω-methyltryptamine as substrates. The slope for the tryptamine data is 0.75 ± 0.08 , and the slope for the *N*^ω-methyltryptamine data is 0.16 ± 0.03 . See "Experimental Procedures" for experimental conditions.

ably assumed to be chemical rather than diffusional (11). The identification of *N*^ω-methyltryptamine (8) as an AANAT substrate with a substantially reduced k_{cat} (~10-fold lower) compared with tryptamine appeared to fulfill the requirement of a poor substrate.

The viscosity of the reaction buffer was manipulated by the addition of sucrose, and the effects on k_{cat} and k_{cat}/K_m with AANAT were examined with *N*^ω-methyltryptamine in the presence of near-saturating acetyl-CoA. The slope of the plot of $k_{cat}\text{-control}/k_{cat}\text{-viscogen}$ versus relative viscosity can give a quantitative estimate of the role of product diffusional release in the kinetic scheme. A slope of 1 indicates a maximal effect (product release is completely rate-determining), and a slope of 0 indicates no effect (product release is much faster than the chemical step). In the experiment, the slope was found to be $+0.16 \pm 0.03$ with *N*^ω-methyltryptamine as substrate and $+0.75 \pm 0.08$ with tryptamine as substrate (Fig. 4).

The slope of a plot of $(k_{cat}/K_m\text{-control})/(k_{cat}/K_m\text{-viscogen})$ can provide information about the "commitment to catalysis" of the substrate as well as information about artifactual effects. High commitment to catalysis indicates that the substrate off-rate is much slower than the (forward) chemical transformation step (10). In the extreme case, in a plot of $(k_{cat}/K_m\text{-control})/(k_{cat}/K_m\text{-viscogen})$ versus the relative viscosity ratio, the slope should approach 1. When the substrate off-rate is much faster than the chemical (forward) step, the slope should approach 0. In this case, the experimental slope with *N*^ω-methyltryptamine as substrate was measured to be -0.14 ± 0.03 (data not shown). With tryptamine as substrate, the K_m values were too small to be accurately measured ($<100 \mu\text{M}$) at high sucrose. The theoretical slope with the poor substrate *N*^ω-methyltryptamine with a high K_m ($>1 \text{ mM}$) and a slow rate would be 0, and the small slope observed may indicate a small, but relatively insignificant non-diffusional effect of sucrose on the enzyme reaction. Overall, the viscosity effects suggest that for the *N*^ω-methyltryptamine reaction, the chemical step is largely rate-determining, whereas for the tryptamine reaction, product diffusional release is the primary rate-determining step.

Rate Versus pH Profiles—Investigations of rate versus pH can sometimes provide information about the properties of ionizable groups that participate in enzymatic catalysis. For example, one way AANAT might accelerate the acetyltransferase reaction is to bind the neutral amine substrate in preference to the protonated ammonium form. This would effectively lead to the experimental observation that the pK_a for the amine substrate would be depressed in the ternary complex.

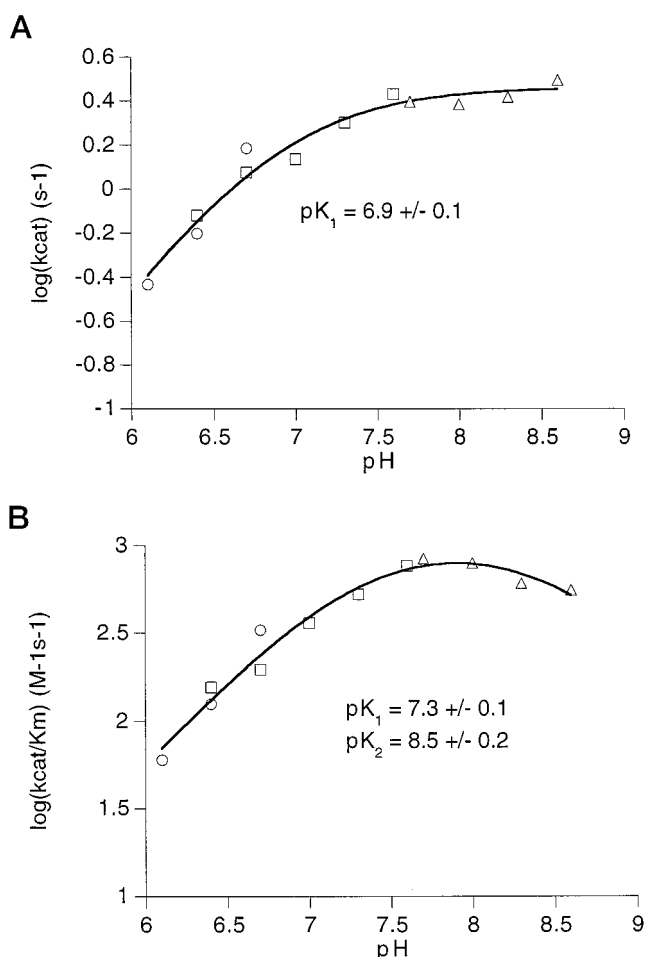


FIG. 5. Profiles of $\log(k_{\text{cat}})$ (A) and $\log(k_{\text{cat}}/K_m)$ (B) versus pH for serotonin *N*-acetyltransferase-catalyzed acetyl transfer with *N*^ω-methyltryptamine as substrate. $\text{p}K_1$ and $\text{p}K_2$ refer to the acid and basic limbs of the profiles, respectively. ○, assays done in Mes; □, assays done in Mops; △, assays done in Epps. See "Experimental Procedures" for experimental conditions and theoretical fits.

Analyses of k_{cat} versus pH generally reveal $\text{p}K_a$ values of ionizable groups in the bound enzyme-substrate complex, whereas plots of $\log(k_{\text{cat}}/K_m)$ versus pH should reflect $\text{p}K_a$ values of ionizable groups present in the free enzyme and/or substrate (12). Measurements of rate versus pH were carried out with the poor substrate *N*^ω-methyltryptamine to maximize the possibility of identifying groups that play a role in the chemical step. The results shown in Fig. 5 indicate that there is an ionizable group with $\text{p}K_a \sim 7$ ($\text{p}K_1$) present in both the free and bound complexes that is required to be in its deprotonated form. This group (with $\text{p}K_1$) facilitates acetyl transfer in its deprotonated form. This is very likely to be an enzymatic group since there are no groups on compound **8** in free solution with such a $\text{p}K_a$ (the secondary amine of compound **8** should have a $\text{p}K_a$ of ~ 10 in free solution). In addition, in the plot of $\log(k_{\text{cat}}/K_m)$ versus pH, there was evidence of a second group ($\text{p}K_2$) with $\text{p}K_a = 8.5$. However, the value of $\text{p}K_2$ is near the upper limit of the pH range where it was technically feasible to perform kinetic studies, somewhat limiting its reliability.

α-Trifluoromethyltryptamine—Information about electronic requirements of the tryptamine substrate is important for understanding catalysis and potentially of great use for inhibitor design. Lowering the amine $\text{p}K_a$ by electronegative atom substitution in the substrate would result in a larger concentration of the neutral amine at pH 7. Depending on the mechanism of AANAT, this could lead to a substrate with a reduced K_m , or it

could lead to an inhibitor since a neutral amine with a reduced $\text{p}K_a$ is generally less nucleophilic than a neutral amine with a higher $\text{p}K_a$. Since the atomic radius of fluorine is only 0.2 Å greater than that of hydrogen, fluorine is close to isosteric with hydrogen, but can significantly change electronic properties because of its strong electronegativity. The use of fluorine substitution in substrate analogs has been a powerful way to probe enzyme mechanism and to generate inhibitors (13). *α*-Trifluoromethyltryptamine (**9**) was synthesized as shown in Fig. 6.

The synthesis of *α*-trifluoromethyltryptamine was achieved following the method of Zimmer and Reissig (6). Commercially available 3-bromo-1,1,1-trifluoroacetone was converted to the corresponding oxime **10** by reaction with hydroxylamine hydrochloride. The oxime was used to generate the vinylnitroso derivative, which was used *in situ* to react in a 4 + 2 cycloaddition and to produce the 3-substituted indole **11**. Reduction of this material with LiAlH_4 led to generation of racemic *α*-trifluoromethyltryptamine (**9**). The *α*-amine $\text{p}K_a$ for compound **9** was measured to be 5.4 ± 0.1 , ~ 4.4 units lower than that of a corresponding unsubstituted amine (e.g. serotonin with $\text{p}K_a = 9.8$ (14)).

In contrast to the isosteric *α*-methyltryptamine, there was no detectable AANAT-catalyzed acetyl transfer to *α*-trifluoromethyltryptamine (at least 1000-fold lower than the k_{cat}/K_m for *α*-methyltryptamine). It was thus tested as a potential competitive inhibitor and was found to be a modest inhibitor with $K_i = 3$ mM, a value similar to the K_m for *α*-methyltryptamine (Table I). This suggests that *α*-trifluoromethyltryptamine can bind about as well as *α*-methyltryptamine to AANAT, but may lack the nucleophilicity to undergo reaction.

DISCUSSION

Homologated Tryptamines—Analysis of the effects on catalytic processing of alkyl chain extension of the ethylamine function of tryptamine contributes to an understanding of the geometric constraints enforced by the AANAT active site. Although a 20-fold decreased catalytic efficiency with the propylamine versus the ethylamine function may seem considerable, it is much less than is observed in perturbing many other enzyme-substrate interactions by the addition of an extra methylene to a functional group undergoing reaction. For example, methylene insertion achieved by an aspartate-to-glutamate substitution in a protein-tyrosine kinase was shown to reduce catalytic efficiency by 10,000-fold (15). Moreover, further extension of the alkyl side chain of the propylamine analog by an extra methylene as present in the butylamine analog (**2**) resulted in a lowering of the k_{cat}/K_m by only 3-fold more. It can reasonably be speculated that relatively weak hydrophobic interactions are involved in holding the tryptamine substrate in place for nucleophilic attack.

The lack of strict side chain recognition allows a more flexible approach to inhibitor design. For example, substitution of the β -position of tryptamine with a halide would be very unstable because it would be in the "benzylic" position (16). The corresponding halide substitution of the β -position in compound **1** would not be complicated by the same problem. The scaffolds of compounds **1** and **2** thus show promise for the development of mechanistic probes and potent inhibitors of AANAT.

Methyltryptamine Analogs—Both *α*-branching and *N*-methyl substitution create steric bulk around the nucleophilic amine without significantly affecting basicity. The methyl substitutions present in compounds **3** and **4** reduce the catalytic efficiency of AANAT processing, but still allow for acetyltransferase reaction. The *R*-enantiomer of compound **3** is much better tolerated than the *S*-enantiomer for enzyme processing.

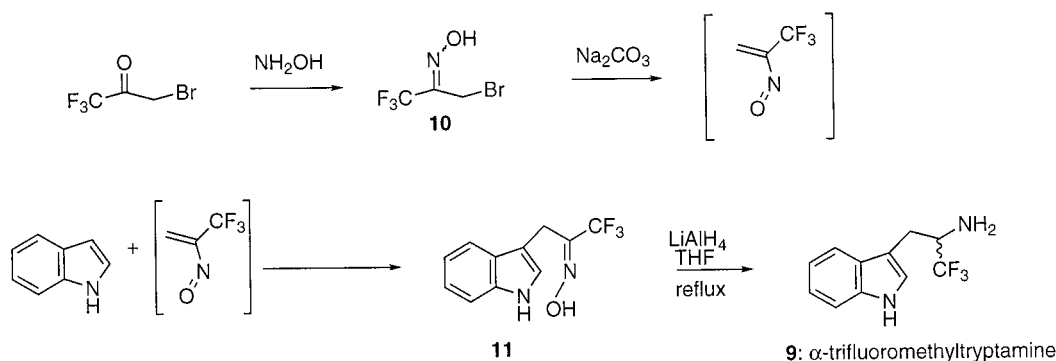


FIG. 6. Synthesis of α -trifluoromethyltryptamine analog (9). THF, tetrahydrofuran.

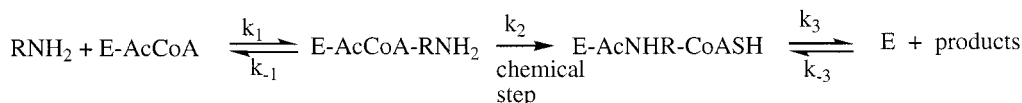


FIG. 7. Kinetic scheme for serotonin *N*-acetyltransferase. RNH₂ corresponds to serotonin or one of the indoleamine analogs used here. AcCoA, acetyl-CoA; AcNHR, *N*-acetyltryptamine.

These studies demonstrate that even a fairly accommodating active site can have well defined stereochemical preferences that need to be considered in designing inhibitors. Furthermore, the stereoselectivity of AANAT could have preparative applications in the production of pharmacologically active α -methyltryptamine congeners (17).

Rate-determining Step(s)—The observation that *N*^ω-methyltryptamine is a poor substrate for AANAT was used along with viscosity experiments to establish that the product release step for standard substrates like tryptamine and serotonin is likely slower than the chemical step. Assuming a kinetic scheme (Fig. 7) in which the ternary complex is fully saturated with substrates and that two steps follow, the chemical step (*k*₂) and the product release step (*k*₃), the effects of viscosity on the steady-state kinetics can be shown to follow those in Equation 4 (8),

$$\left(\frac{k_{\text{cat-control}}}{k_{\text{cat-viscogen}}} \right) \text{slope} = \frac{k_2}{(k_2 + k_3^\circ)} \quad (\text{Eq. 4})$$

where *k*₃[°] is the rate constant for the release of products in the absence of viscogen. Neglecting potential small artifactual effects, a quantitative interpretation of the slope of +0.75 would indicate that *k*₂ is approximately three times greater than *k*₃[°] for the substrate tryptamine. Since the overall *k*_{cat} is 27 s⁻¹ with tryptamine, the *k*₂ would be 108 s⁻¹ and the *k*₃[°] would be 36 s⁻¹ under these circumstances. At this time, the nature of the rate-determining step is not established and could include CoASH release, *N*-acetyltryptamine release, or an enzyme conformational change. Let us make the plausible assumption that this rate constant (*k*₃) is identical for enzyme reactions with tryptamine and *N*^ω-methyltryptamine (8). Given that the *k*_{cat} with this substrate was measured to be ~2.5 s⁻¹, based on Equation 4, the slope with compound 8 as substrate would actually be expected to be about +0.07 rather than 0, assuming the absence of non-diffusional sucrose effects. The actual measured value of +0.16 falls reasonably close to this theoretical value of +0.07. The 0.09 difference in slope between theory and experiment could be subtracted from the slope obtained with tryptamine as substrate as a correction factor for non-diffusional effects of sucrose. This 0.09 difference is almost within the error of the slope measurement (0.75 ± 0.08) for this substrate, so the estimated rate constants for tryptamine would hardly be affected.

Based on the calculated rate constants of *k*₂ and *k*₃ for tryptamine, the *K*_d for tryptamine binding to the

AANAT-acetyl-CoA complex would likely be significantly greater than the *K*_m for tryptamine (18). Such a relationship needs to be borne in mind when considering the affinity of a series of tryptamine analogs for the enzyme. Another important point is that mechanistic studies designed to probe the chemical step catalyzed by AANAT may be simpler to interpret by studying the poor substrate *N*^ω-methyltryptamine.

Activation of the Amine Nucleophile—That α -trifluoromethyltryptamine was not a substrate for the enzyme could imply that reduced nucleophilicity of the neutral species compared with neutral tryptamine slows the rate of the chemical step. The straightforward possibility that the fluorines prevent binding of the analog seems to be unlikely since the *K*_i for racemic compound 9 is similar to the *K*_m for racemic α -methyltryptamine (7). For racemic α -methyltryptamine, the *K*_m and *K*_d are probably similar since most likely *k*₂ < *k*₃, and *k*₁ and *k*₋₁ are likely to be fast for this substrate. Although the fluorines may themselves lead to reduced affinity through repulsive electronic interactions (19), the inability of compound 9 to bind more tightly to AANAT also suggests that the enzyme may preferentially bind the charged protonated ammonium ion rather than the neutral amine. The pH-rate analysis with the substrate *N*^ω-methyltryptamine also favors this possibility based on the similarity of p*K*₁ in the log(*k*_{cat}) and log(*k*_{cat}/*K*_m) plots. These similarities are evidence against the substrate amine p*K*_a dropping in the active site since an additional ionizable group with p*K*_a < 8 might have been expected in the log(*k*_{cat}) profile.² The behavior with AANAT differs from that of the amine substrate in the glutathionyl-spermidine synthetase-catalyzed reaction, in which the neutral amine nucleophile, generated by fluorine substitution, seems to bind more tightly to the enzyme than the charged species (20). With AANAT, the evidence favors a mechanism in which the protonated amine is deprotonated concomitant with attack on the acetyl group of acetyl-CoA. The lack of reactivity of the trifluoromethyl compound likely highlights the importance of nu-

² An alternative interpretation of the rate versus pH profiles is that two different ionizable groups are being titrated in the *k*_{cat} and *k*_{cat}/*K*_m plots that coincidentally have p*K*_a values near 7 in the free enzyme and enzyme-substrate complex, respectively. For this to be true, the p*K*_a values of each of the two groups would have to shift dramatically or be offset by other ionizable groups upon substrate binding. This model is not favored because of its complexity, but cannot be formally ruled out at this time.

cleophilicity in the enzyme reaction. Assume that the β_{nuc} for the chemical step is 0.8, similar to related chemical model reactions (21). The difference in $\text{p}K_{\text{a}}$ values of the amines of the trifluoromethyl-substituted compound **9** compared with α -methyltryptamine (**7**) is 4.4 units, so the predicted rate reduction due to decreased basicity of compound **9** would be $10^{3.5}$ -fold. Disruption in binding and/or orientation due to fluorines could further reduce the rate. Even a $10^{3.5}$ -fold reduction is below the background experimental detection level (10^3 -fold lower than for compound **7**) and is consistent with the experimental inability to detect turnover with substrate **9**, assuming a transition state with $\beta_{\text{nuc}} = 0.8$. Arylamines such as aniline ($\text{p}K_{\text{a}} = 4.7$) have been reported not to be substrates for AANAT, whereas the related compound phenethylamine ($\text{p}K_{\text{a}} = 9.8$) is an effective substrate (1). Based on the arguments above, the decreased nucleophilicity of arylamines would be insufficient to permit efficient AANAT-catalyzed acetyl transfer.

The pH-rate analyses did reveal an ionizable group with $\text{p}K_{\text{a}} \sim 7$ ($\text{p}K_1$) for both the free and bound enzyme complexes.² Although the role and identity of this residue are not known at present, a reasonable speculation is that it might be an enzyme histidine imidazole. Such a $\text{p}K_{\text{a}}$ would be consistent with a previously proposed histidine in catalysis whose role was suggested based on mutagenesis studies (1). Further analysis will be necessary to clarify this connection.

Conclusions—Recombinant sheep AANAT catalyzes acetyl transfer to a diverse array of tryptamine analogs. This relatively liberal acceptance of amine substrates offers hope for the generation of potent AANAT inhibitors. Despite this flexibility, AANAT can show stereoselectivity for α -branched substrates. Such behavior is useful knowledge for inhibitor design. It may also have utility in the preparation of enantiomerically pure tryptamine analogs. With physiologic substrates, it is likely that the chemical step is fast and that product release is largely rate-determining. This kinetic feature needs to be taken into account in the interpretation of mutagenesis and other perturbations of catalytic mechanism and substrate affinity. It could also be important in studies of biological regulation of acetyl-

transferase activity. Based on studies with the α -trifluoromethyltryptamine analog and pH-rate profiles, the favored model for the catalytic mechanism of AANAT involves binding of the positively charged serotonin ammonium species, which subsequently becomes deprotonated and converted to a strong nucleophile during catalysis.

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