Substrate Specificity and Inhibition Studies of Human Serotonin N-Acetyltransferase*

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Arylalkylamine N-acetyltransferase (AANAT) catalyzes the reaction of serotonin with acetyl-CoA to form N-acetylserotonin and plays a major role in the regulation of the melatonin circadian rhythm in vertebrates. In the present study, the human cloned enzyme has been expressed in bacteria, purified, cleaved, and characterized. The specificity of the human enzyme toward substrates (natural as well as synthetic arylethylamines) and cosubstrates (essentially acyl homologs of acetyl-CoA) has been investigated. Peptide combinatorial libraries of tri-, tetra-, and pentapeptides with various amino acid compositions were also screened as potential sources of inhibitors. We report the findings of several peptides with low micromolar inhibitory potency. For activity measurement as well as for specificity studies, an original and rapid method of analysis was developed. The assay was based on the separation and detection of [3H]acetylarylethylamine formed from various arylethylamines and tritiated acetyl-CoA, by means of high performance liquid chromatography with radiochemical detection. The assay proved to be robust and flexible, could accommodate the use of numerous synthetic substrates, and was successfully used throughout this study. We also screened a large number of pharmacological bioamines among which only one, tryptamine, behaved as a substrate. The synthesis and survey of simple arylethylamines also showed that AANAT has a large recognition pattern, including compounds as different as phenyl-, naphthyl-, benzothienyl-, or benzo-furanyl-ethylen derivatives. An extensive enzymatic study allowed us to pinpoint the amino acid residue of the pentapeptide inhibitor, S 34461, which interacts with the co-substrate-binding site area, in agreement with an in silico study based on the available coordinates of the hAANAT crystal.

Melatonin (5-methoxy-N-acetyltryptamine) is a pineal hormone that modulates a variety of endocrinological, neurophysiological, and behavioral functions in vertebrates (1). It is involved in the regulation of circadian rhythms and in the reproduction of photoperiodic species (2). The chronobiotic effects of melatonin in humans have been mainly studied in circadian rhythm sleep disorders (3). Moreover, alterations of the melatonin profiles have been reported in other biological rhythm disorders (3). Melatonin exerts its effects through at least three targets: 2 receptor subtypes, MT1 and MT2, and a binding site, MT3 (4). The two first ones have been cloned (5–6) and their pharmacological effects largely studied, and several specific and potent ligands (7–9) discovered. The MT3 subtype is still a putative binding site under intensive research from purification attempts to pharmacological characterizations (10–11). Since melatonin is implicated in several types of mild to severe pathologies, including mood disorders (3, 12), it is considered a valuable therapeutic target. Beside the classical search for agonists and antagonists of the melatonin receptors, a series of programs was launched that was aimed at the control of the levels of circulating melatonin. Indeed, melatonin biosynthesis is catalyzed by a series of enzymes, the penultimate of which in the synthesis cascade, serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT, EC 2.3.1.87), catalyzes the rate-limiting step. It has been shown that large increases in its activity are responsible for large increases in circulating melatonin levels in vertebrates (13–14). AANAT regulates melatonin biosynthesis by controlling the production of N-acetyl-5-hydroxytryptamine from serotonin (5-hydroxytryptamine) and acetyl-CoA (15). In the next step, the conversion of N-acetyl-5-hydroxytryptamine to melatonin is catalyzed by hydroxyindole-O-methyltransferase. However, this transferase is expressed at relatively constant levels, and the rate of this step is regulated by the availability of N-acetyl-5-hydroxytryptamine. Furthermore, the level of AANAT is controlled by a catabolic system involving light, cAMP, and a proteasome multienzyme complex (16). Therefore, AANAT appears to be a major target for the control of the pineal hormone circulation.

Sheep AANAT (oAANAT) has been cloned and overproduced in Escherichia coli (17). Once purified, this recombinant ovine...
enzyme has been crystallized and the crystal coordinates published (18–19). Knowledge of the human enzyme has progressed more slowly, although Coon et al. (20) have reported the structure of the human gene encoding this enzyme. Studies of the enzyme distribution outside the retina and pineal gland have shown its presence in different peripheral tissues such as gastrointestinal tract (21), testes (22), and ovaries (23) as well as in other limited brain regions (24–26). Substrate and cosubstrate specificities of the ovine enzyme have been only marginally addressed so far (14, 27–31) and served as the sole basis for the characterization of AANAT from other species, rat (32), rabbit (33), ox (34), and hamster (35).

In the present work, hAANAT (EC 2.3.1.87) was expressed as a fusion protein in a bacterial system, and the protein was purified and cleaved, leading to large amounts of partially pure biological material. The human enzyme was then characterized biochemically and compared with the ovine form. The search for potent substrates and inhibitors was carried out in a large screening process that included synthetic and natural ligands. Two techniques are available for the measurement of AANAT activity, the highly sensitive, extraction-based assay of Deguchi (27) and the chromatographic assay of Thomas et al. (36). The latter has been developed for and is limited to fluorescent compounds (37), whereas the former is difficult to automatize. Since our program required a relatively high throughput capacity, we developed an HPLC assay for substrate and inhibitor screening running less than 6 min per sample. By using labeled acetyl-CoA, it was possible to test any type of ethylenamine derivative as potential substrate, regardless of its water/chloroform repartition, in contrast to the Deguchi extraction assay (27). Large tri-, tetra-, and pentapeptide combinatorial libraries were also screened as potential sources of inhibitors, leading to active leads in the micromolar range, displaying either pure or mixed competitive kinetic behavior. Molecular modeling and structure-activity relationship studies made it possible to pinpoint the amino acid residue of the pentapeptide inhibitor S 34461 that interacts with the cosubstrate-binding site. The present report is the first one on the biochemical description of the human enzyme. This study reveals some unexpected differences with the ovine enzyme. Finally, synthetic substrates of AANAT and new inhibitors are discovered and described.

MATERIALS AND METHODS
Expression and Production of hAANAT

The human arylalkyl N-acetyltransferase cDNA coding region (kindly provided by Dr. D. C. Klein and Dr. S. L. Coon, National Institutes of Health, Bethesda) was inserted into the bacterial expression vector pGEX-4T (Amersham Pharmacia Biotech). The E. coli strain BL21(DE3)pLyS3 was transformed with the resulting plasmid and grown overnight at 37 °C in Luria Broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The culture was diluted 1:25 in fresh medium and grown at 37 °C, until it reached an absorbance at 595 nm of 0.7. Isopropyl–β-D-galactopyranoside was then added to a final concentration of 0.2 mM, and the culture was maintained at 24 °C for 6 h. The cells were harvested by centrifugation (5,000 × g, 4 °C, 10 min), frozen in dry ice, and stored at −80 °C until further use.

Purification of hAANAT

All the procedures were performed at 0–4 °C. Approximately 10 g of a frozen bacteria pellet expressing GST-hAANAT was thawed in 40 ml of 2× phosphate-buffered saline containing 10 mM dithiothreitol (DTT), a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals, 1 tablet/50 ml), and with or without the detergent Tween 85 (at 1% v/v). The thawed bacterial suspension was sonicated (setting, 70/100, probe 1 cm diameter) eight times for 1 min duration, with 1-min interval between each sonication. The preparation was immediately centrifuged (20,000 × g, 20 min), and the supernatant was slowly (40 ml/h) passed through a glutathione-Sepharose column (Amersham Pharmacia Biotech, 10 ml packed volume), equilibrated with buffer A (2× phosphate-buffered saline, pH 6.9, containing 10 mM DTT).

HPLC Method—This new assay for the AANAT activity was based upon reverse-phase HPLC using either absorbance or radiochemical detection of N-acetylserotonin. The reaction mixture contained 10 μl of enzyme (1 μg for the human enzyme, but 10–50 times less for the ovine enzyme), in a phosphate buffer (50 mM sodium phosphate, pH 6.8, containing 500 mM NaCl and 2 mM EDTA), 10 μl of [3H]acetyl-CoA (129GBq/mmol), 1 mM acetyl-CoA, 4 mM serotonin, in a final volume of 100 μl. After incubation of 30 min at 37 °C, it was stopped by the addition of 50 μl of 10% trichloroacetic acid solution. Thirty μl of this solution were analyzed by reverse-phase HPLC using a Platinum EPS C8, (53 × 7 mm, Altich, France) column on a Hewlett-Packard 1100 system. The column was eluted with a linear gradient of 5–95% acetonitrile in H2O, 0.1% trifluoroacetic acid at a flow rate of 2 ml/min for 5 min. While the separation took about 7.5 min, efficient mass spectrometry detection required longer separation times (10 min) as did also more hydrophobic substrates with higher gradient curves (30 min). This type of separation was obtained in the same buffer using a different polymeric analytical column (C4 ASTEC, 4.6 × 150 mm, CIIL, Cluzeau, Ste-Foy-la-Grande, France). The column was eluted with a linear gradient 5–95 or 0–100% acetonitrile in H2O, 0.1% trifluoroacetic acid at a flow rate of 1 ml/min for 10 or 30 min. The C4 Asteck polymeric column and tritiated serotonin (NEN Life Science Products, 1 TBq/mmol, 10 μl in incubation with cold serotonin 4 μmol final) were used to study other cosubstrates than acetyl-CoA. HPLC gradient and buffer were the same as described above for the 15-min run. The radioactivity was followed on-line after addition of the scintillation mixture (2 ml/min) using a Berthold detector (EGG, Bad Wildbad, Germany).

Kinetic Studies

Except when otherwise noted, all the measurements were done using the HPLC assay. Basically, the affinity data have been collected using the classical process described by Segel (38) for bisubstrate enzymes as applied on this transferase (30, 31) or on other ones (39). For the apparent Km value, the substrate concentrations were varied from 0.06 to 4 mM, whereas the cosubstrate (acetyl-CoA) concentration was 1 mM. For apparent Kapp values of acetyl-CoA, a saturating concentration of 4 mM was used for the substrate (usually, serotonin). Although the rule is that the saturating concentration should be 10 times the Kapp value (38), for most of the natural bioamine substrates of AANAT, this concentration could not be reached (10 μM). For real Km determinations, experiments were also conducted according to the guidelines given by...
Segel (38). At least five concentration points were routinely used, and the experiments were repeated three times. Finally, for treatment of the data, double-reciprocal plots were used as the most accurate method when less than 10 concentration points are available.

**Mass Spectrometry Studies**

Mass spectra were recorded on a TSQ 7000 triple quadrupole instrument (Finnigan, San Jose, CA) operating in positive atmospheric pressure chemical ionization mode (APCI+). The API vaporizer was operated at 400 °C with a capillary temperature of 250 °C. Nitrogen was used as auxiliary gas (35 mL/min) and as sheath gas (70 pounds/square inch). The corona voltage was 5 kV. All spectra were acquired in centroid mode. Collision-induced dissociation (CID) experiments were carried out by setting Q1 to pass only the ions of interest (IM + 1H) ± 0.3 Da in 0.5 s), inducing collisions in Q2 and scanning Q3 from 19 to 600 Da with a scan time of 1 s. MS/MS analyses were performed with argon as collision gas at a pressure of 1 millitorr and a dissociation offset of 20 eV. Single ion-monitored (SIM) parameters were 0.9 Da/s scan time and >0.3-Da scan window. LC separations for the LC/MS experiments were performed using a Hewlett-Packard 1090 binary pump equipped with a UV detector. The detection wavelength was set at 210 nm. The samples passed through a 20-µl injection loop, into the ASTEC polymeric C4 column (see above) at 1 ml/min flow rate and directly into the mass spectrometer. The eluents were as follows: eluent A, 0.01% trifluoroacetic acid in deionized water, and eluent B, 0.01% trifluoroacetic acid in acetonitrile, and the gradient was built from 95 to 50% of eluent A in 30 min.

**Peptide Library Synthesis**
The libraries were synthesized using a robotic instrument built around a Zymark arm (40). The robot was able to handle 1 g of resin with 2 × 10⁶ beads per reactor, therefore ensuring a high ratio of the number of beads to the number of tetrapeptides (41). The robot automatically handled the main steps of the mix and split procedure (42) using the Fmoc strategy for peptide synthesis on solid phase (43). The libraries were prepared from sets of 24 different amino acids depending on the library types. The exact amino acid composition of the tripeptide library was at position 1: γ-aminobutyric acid, 4-aminoquinolylalanine, Arg, β-alanine, benzo thienylalanine (Bta), cyclohexylalanine (Cha), 4-chlorophenylalanine (Clp), 4-fluorophenylalanine (Fpa), Glu, Gly, Glu, His, Lys, methionine sulfoxide, 4-nitrophenylalanine (Nip), nor leucine (Nle), Phe, phenylglycine, 3-phenylalanine, tert-butylglycine, thienylalanine, Thr, Tyr, and O-methyltyrosine. This "optidiverse" set of amino acids displays a high fingerprint diversity, compared to the mixed affinity column and neither was the chaperone (Fig. 1, lane 7), whereas both thrombin and GST were retained (Fig. 1, lane 5). In order to find conditions under which not only the enzymatic activity but also the amount of solubilized enzyme was affected, several detergents were tested on the bacterial pellet at a concentration of 0.1% (v/w): deoxycholate, lauryl sulfate, CHAPS, Triton X-100, Triton X-114, Triton X-405, Tween 70, Tween 85, Brij 58, and Igepal CA630. The results indicated that, depending on the type of detergent, the enzymatic reaction was either boosted (200% for Tween 85) or inhibited (3% remaining activity for lauryl sulfate). A dose-dependent study showed a maximal value of the enzyme activity at the detergent (Tween 85) concentration of 1% (v/w). The detergent not only helped to solubilize the enzyme from the bacterial pellet but also reduced

**Synthesis of Analogos**

The most active single peptides and several analogs were either resynthesized by solid phase synthesis using an Fmoc strategy on the Zymark robot in the parallel mode (no mixing step). The final compound was obtained after cleavage and deprotection in 95% trifluoroacetic acid in the presence of scavengers. They were purified by preparative HPLC and lyophilized. Peptides were at least 95% (HPLC) pure. Furthermore, a series of single analogs were synthesized, including the five peptides of an Ala scan, the five peptides of a Gly scan of the pentapeptide inhibitor S 34461, and the five tetrapeptide corresponding to the successive deletion of each amino acid residue in S 34461.

**Synthesis of Chemicals**

A series of amines was synthesized in order to find out which of the ethylamine derivatives was recognized by hAANAT as substrates. SD 219 was synthesized as described by Fournier and Boyer (48), SD 236 by Chan et al. (49), SD 552 by Sam et al. (50), SD 715 by Khalil and Cole (51), S 24202 and S 5065 by Lesieur et al. (52), S 23953 and S 24495 by Lesieur et al. (53), S 24198 by Miyamoto et al. (54), and S 24192 by Fourmaintraux et al. (55).

**Molecular Modeling**
The models of the human AANAT enzyme with the pentapeptide inhibitor S 34461 (Trp-Nip-Val-Ile-Nal1) are based on the crystal structure of sheep AANAT complexed with the bisubstrate analog SD 715 (18). The model of the human AANAT (19) was generated using the homology building routines PHIPPS and REFL in Whatif (56). This model was energy minimized with SYBYL version 6.5 (Tripos Associates, Inc., St. Louis, MO) by applying twice conjugate-gradients minimization (100 steps each). The MMFF94 force field within SYBYL was used for all energy calculations. Ligand docking was done using the molecular dynamics simulated annealing algorithm within SYBYL. The ligand was first docked manually in the binding site with all peptide bonds in trans configuration and using the key ligand-enzyme interaction sites of the bisubstrate analog as reference. The three possible conformers (i.e., the three possible arrangements of either Trp, Nip, or Nal1 in positions 1, 2, and 5 of the peptide inhibitor) with the tryptamide fragment of the bisubstrate analog were considered. For each of these initial docking conformations, 25 constrained simulated annealing structures were calculated by applying 2-ps molecular dynamics simulation at 2000 K followed by 2-ps exponential cooling to 0 K. The protein structure was kept fixed while the ligand was flexible. Additional distance-range constraints (force constant 20 kcal/molÅ²) were included in order to conserve the interactions analogous to those of the tryptamide fragment of the bisubstrate analog, i.e. 1) the amide group NH and C=O bond to the C=O of Met159 and the NH of Leu159 (1.5-3.5 Å); 2) the centroid of central aromatic ring bond to the centroids of the rings of Phe36 (3.4-5.4 Å), Phe44 (2.8-4.8 Å), and Phe18 (4.2-6.2 Å). The absolute best position found for each ligand with trans-peptide bonds were finally energy minimized using the conjugate-gradients algorithm (100 steps) without restraint to give the final models.

**RESULTS**

**Expression and Purification of hAANAT—** The expression of the GST-hAANAT encoding gene in E. coli resulted in the production of an active soluble GST-hAANAT (>10 mg/liters of culture). Partial purification was obtained by glutathione affinity chromatography, and a 47-kDa band identified by SDS-PAGE (Fig. 1, lane 4) was shown to correspond to GST-hAANAT contaminated with a bacterial chaperone. hAANAT was cleaved by thrombin (Fig. 1, lane 5) to give both a 21-kDa hAANAT (Fig. 1, lane 7) and a 26-kDa GST band (Fig. 1, lane 6) after mixed affinity chromatography on benzamidine/glutathione-agarose. Both fractions were contaminated with the chaperone. Western blot analysis of pure and cleaved GST-hAANAT with anti-GST antibodies revealed a GST band at 47 kDa corresponding to GST-hAANAT and a 26-kDa band corresponding to GST (not shown). hAANAT was not adsorbed on the mixed affinity column and neither was the chaperone (Fig. 1, lane 7), whereas both thrombin and GST were retained (Fig. 1, lane 6). In order to find conditions under which not only the enzymatic activity but also the amount of solubilized enzyme from the bacterial pellet was enhanced, several detergents were tested on the bacterial pellet at a concentration of 0.1% (v/w): deoxycholate, lauryl sulfate, CHAPS, Triton X-100, Triton X-114, Triton X-405, Tween 70, Tween 85, Brij 58, and Igepal CA630. The results indicated that, depending on the type of detergent, the enzymatic reaction was either boosted (200% for Tween 85) or inhibited (3% remaining activity for lauryl sulfate). A dose-dependent study showed a maximal value of the enzyme activity at the detergent (Tween 85) concentration of 1% (v/w). The detergent not only helped to solubilize the enzyme from the bacterial pellet but also reduced
the chaperone-hAANAT interaction and led to a final preparation that contained less bacterial contaminant (Fig. 1, lane 8). By comparing the band staining on the gel lanes by densitometry, it was possible to evaluate the respective amounts of the GST-AANAT and of the chaperone (Fig. 1). The ratio between the contaminant and GST-hAANAT without Tween 85 was \(-1:1\) (lane 4), and in the presence of the detergent, this ratio dropped to \(-1:4.5\) (lane 8). The contaminant was therefore present in far less quantity in the presence of Tween 85. Under those experimental conditions, the main protein present in the preparation was GST-hAANAT. All the experiments done to compare enzyme kinetics between the fused and the cleaved AANAT were performed with this material.

**HPLC Assay Development**—As done in the past in this Institute for several other enzyme types (tyrosine protein kinases (57), N-myristoyltransferase (58), S-farnesyltransferase (59), UDP-glucuronosyltransferase (60)), a new HPLC assay for AANAT activity was developed. Although the standard assay for AANAT (27) was found to be highly reliable, it was not easy to automate and was therefore poorly adapted to a screening strategy. Fig. 2, A and B, shows a typical chromatogram obtained with a GST-hAANAT-purified fraction, incubated 30 min at 37 °C with serotonin and acetyl-CoA as described under “Materials and Methods” (short time assay). Elution could be easily followed both by absorption or by on-line radioactivity quantification. \[^{3}H\]Acetyl-serotonin (Fig. 2, A and B, peak b) produced during the incubation of the enzyme with serotonin and \[^{3}H\]Acetyl-CoA was identified. Several controls were run, including the use of another labeled compound, \[^{3}H\]serotonin as well as a wide panel of potential bioamine substrates. Finally, the products of the reaction using either serotonin (not shown) or phenylethylamine (PEA) as substrate (Fig. 2C) were positively identified by LC/MS. Serotonin, PEA, and their N-acetylated homologs are commercially available, and their retention times in the HPLC analysis are known. UV spectra were recorded simultaneously with the real time display of MS chromatograms for the four analyzed compounds. Under these conditions, it was not possible to get sensitive UV signals that could be useful in the characterization of the compounds. To reduce the background and provide more sensitivity, experiments were run in the SIM mode. Fig. 2C shows SIM chromatograms of an incubation of PEA (4 \(\mu\)M) with acetyl-CoA and hAANAT. Unchanged PEA (Fig. 2C, peak d, \([M + H]^+\) at \(m/z\) 122) eluted at a retention time of 4.1 min, and N-acetyl-PEA at 10.0 min (Fig. 2C, peak e, \([M + H]^+\) at \(m/z\) 164). In order to get more information on structural data, compounds were examined by CID mass spectrometry after incubation with hAANAT. Under CID conditions, compounds had a main fragmentation corresponding to the elimination of NH\(_3\) for PEA and serotonin and to the loss of AcNH\(_2\) for the two N-acetylated compounds. Indeed, these experiments confirmed the N-acetylation as the reaction catalyzed by the cloned enzyme. Further experiments were conducted to confirm the nature of the products of the reactions whenever “new” amines were incubated as potential substrates with hAANAT. By using the HPLC assay, quantification could easily be done using \[^{3}H\]acetyl-CoA by simple measurement of the area under the product peak. Comparison of the data obtained with this HPLC assay with those generated with the extraction assay showed similar activities (not shown) agreeing at least within a factor 2, 1.33 and 1.66 mol/min/mg protein for activities measured with the fused hAANAT and 16.6 and 33.3 \(\mu\)mol/min/mg protein for the pure hAANAT. Similar data with both methods were obtained with the sheep enzyme as was also reported in the literature (17, 19, 30).

**Biochemical Characterization**—The conditions of incubation...
were checked, as presented in Fig. 3. The assay ran linearly for up to 30 min (Fig. 3A). The enzyme activity was linear from 150 ng up to 7 mg of enzyme per assay and plateaued thereafter (Fig. 3B). Since the human enzyme behavior proved to be slightly different from other forms, particularly the ovine enzyme, according to several parameters (stability, solubility, specific activity, specificities toward both substrates and inhibitors), the kinetic constants were checked on the preparations before and after thrombin cleavage. Indeed, Table I shows great similarity between GST-AANAT and cleaved AANAT specificities regarding substrate affinities (91% similarity for serotonin and 97% for tryptamine) and cosubstrate affinity (96% for acetyl-CoA). Specific activities were slightly different, the fusion protein being 10 times less active than free AANAT. This difference was more pronounced when the Deguchi assay (27) was used. Despite these differences in activities, the fused proteins were used throughout the following experiments.

The specificity of the enzyme toward its cosubstrate was also studied. A series of commercially available natural acyl-CoAs ranging from C2 to C16 acyl chains were tested. Only acetyl- and propionyl-CoA were effective cosubstrates of the enzyme with relative activities being ~50 (propionyl-CoA) and ~10% (butyryl-CoA) of the activity recorded under the same conditions with acetyl-CoA. The use of the HPLC assay with [3H]serotonin permitted us to visualize the products of the reaction and to measure their retention times. All other derivatives proved to be inefficient in acylating the substrate. Another set of experiments was designed, in order to study the possible inhibiting capacity of acyl-CoA derivatives. By using a series of

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Representative chromatographic profile of the products of the reaction catalyzed by arylalkylamine N-acetyltransferase as estimated by radioactivity, UV, or by LC-MS. A and B, acetylation of serotonin. Human arylalkylamine N-acetyltransferase was incubated as described under “Materials and Methods” with 4 mM serotonin, 1 mM acetyl-CoA, and 10 µM [3H]acetyl-CoA (4 Ci/mol) for 30 min at 37 °C. The reaction was stopped and the incubation medium injected in a HPLC system equipped with a C4 Astec column. UV absorption at 210 nm (B) and radioactivity were measured on-line (A) after addition of 2 ml/min scintillation mixture by a slaved pump. The chromatogram is representative of the assay in real time conditions. Peak identifications: a, [3H]acetyl-CoA; b, N-[3H]acetyl-serotonin; and c, serotonin. C, acetylation of PEA: LC-MS analysis. Peak d, unchanged PEA, and peak e, N-acetyl-PEA. Note the short separation time of 5 min for the standard routine assay (A and B), different from the LC/MS profile, 15 min (C).

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Assessment of the LC assay conditions of the human arylalkylamine N-transferase. A, incubation conditions were as described under “Materials and Methods” except that they were all started at the same time and stopped at the indicated times. Aliquots were injected in the HPLC system. The area under the peak was plotted versus the incubation time. B, various quantities of the fusion protein were incubated as described. The resulting areas under the peak were plotted versus the enzyme amount. Results are means ± S.E. of three experiments.

**TABLE I**

| Enzyme    | Acetyl-CoA | Serotonin | Tryptamine |
|-----------|------------|-----------|------------|
| GST-hAANAT| 530 ± 38   | 1350 ± 115| 880 ± 75   |
| hAANAT    | 550 ± 22   | 1235 ± 75 | 910 ± 36   |
| oAANAT    | 178 ± 13a  | 125 ± 14a | 110 ± 20b  |
| GST-oAANAT| 300d       | 240 ± 30d | 170 ± 10d  |
| ND        | 234 ± 16   | 335 ± 10  |

a DeAngelis et al. (30).

b On partially purified enzyme from sheep pineal gland, Voisin et al. (15).

c Coon et al. (17).

d Khalil et al. (31).
such compounds, with acyl chains from C₄ to C₂₀. IC₅₀ values were measured using the HPLC assay (Fig. 4). A slow decrease of the IC₅₀ absolute values was found when going from C₄ to C₈ acyl chains, followed by a marked decrease of the IC₅₀ to the high nanomolar range, reaching 420 nM for decanoyl acetyl-CoA.

As a control, some of the corresponding fatty acids were tested without their CoA conjugate. The inhibition recorded with the free acid was in the millimolar range for all those tested, thus ruling out a detergent-like effect of the alkyl chains of the fatty acids.

**Search for Natural Substrates**—No information on the substrate specificity of hAANAT is so far available, although several studies were published on the specificity of AANAT from other origins, particularly sheep and rat (15, 27–31, 51). A large panel (see list in the legend of Table II) of bioamines was tested among which only five behaved as substrates of the enzyme: tryptamine, 5-methoxytryptamine, serotonin, and phenylethylamine (Table II). All other bioamines did not behave as potent substrates, even those derived from PEA such as dopamine and amphetamine.

Since hAANAT belongs to the A/B superfamily of acetyltransferases, which includes the histone N-acetyltransferases (18), a series of purified, commercially available histones was also checked as potential substrates using an electrophoresis/autoradiography assay. However, neither histone H1a, H2a, H2b, H3 nor H4 incorporated radioactivity under our experimental conditions.

**Search for Synthetic Substrates**—A number of amines, used as drugs or pharmaceutical agents, were tested as substrates of hAANAT. None of the following compounds were substrates (or inhibitors), at 1 mM, of the hAANAT: 2-(p-chlorophenoxoy)-2-methylpropionic acid ethyl ester, 4-aminopyridine, amiloride, aminophylline, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), benzamide, clozapine, codeine, epibatidine, hydrazine, memantine, minoxidil, p-aminobenzoic acid, p-aminoalicyclic acid, phaclophen, procainamide, sepiapterin, spermidine, spermine, thyamine, and uracil.

Similar data were reported for the cloned ovine enzyme (17). Only one compound, a synthetic amine related to PEA, tranylcypromine (±)-trans-2-phenylcyclopropylamine) was recognized and acetylated by the enzyme with a $V_{\text{max}}$ of 438 ± 27 μmol/min/mg of protein. Several unsuccessful attempts were also made to acetylate enzymatically N⁰-methyldtryptamine (31).

Furthermore, the search for new synthetic structures able to be recognized by the enzyme led to the synthesis of a series of simple 2-arylethylamines. By using the HPLC assay, the catalytic reaction could be analyzed with any type of compounds, regardless of their lipophilicity and solubility in chloroform, whereas the assay of Deguchi (27) depends on these parameters. Bicyclic compounds were synthesized, including naphthyl-, biphenyl-, benzofuryl-, benzothiophenyl-, and tetralinylethylamines from which a representative series was selected in Table III. 2-(4-Biphenyl)ethyl amine did not behave as a substrate, whereas tetralinylethylamine derivatives were poor substrates, compared with the indole-based natural substrate, serotonin. The three other series (benzofuryl, naphthyl, and benzothiophenyl derivatives) included good substrates of AANAT. Surprisingly, they were even better substrates than serotonin with $K_m$ values ranging from 40 to 500 μM, whereas tryptamine and serotonin displayed $K_m$ values in the 800–2500 μM range. Nevertheless, none of the alternative synthetic substrates had better rate constants than PEA (159–4000 μmol/min/mg protein) or tryptamine (800 μmol/min/mg protein), whereas for serotonin, it was comparable to the aryl bicyclic compounds (200–300 μmol/min/mg protein). Incidentally, none of them inhibited the AANAT activities, whether PEA or serotonin were used as a substrate of the enzyme.

**Search for Synthetic Inhibitors**—AANAT is a major target for the control of melatonin level. Indeed, specific inhibitors of this enzyme will impair the biosynthesis of melatonin and rapidly decrease the level of the circulating pineal hormone. It is therefore desirable to find potent, selective inhibitors able to interfere with the enzyme activity in situ. Systematic screening included the synthesis and deconvolution of peptide libraries. This approach had provided several new inhibitory structures from tri-, tetra-, and pentapeptide libraries containing 2,880–331,776 members. The pentapeptide library was a biased library in which the N-terminal amino acid was tryptophan and comprised “only” four random positions.

The first round of deconvolution of the tripeptide libraries led...
to the tryptophan analog, benzothienylalanine, as the best residue in position 1 (not shown). Position 2 was composed of various analogs of the indole core of melatonin: Nal1, Nal2, Trp, Bta, and the PEA analog, tyrosine, based on published observations (29). 1-Naphthylalanine was the best amino acid in position 2, whereas the final round gave several possibilities among which Met and Nle were selected. Indeed, the final peptides S 34578 (Bta-Nal1-Met) and S 34381 (Bta-Nal1-Nle) inhibited the enzyme activity with IC50 values of 176 and 159 μM, respectively (see Table IV).

The tetrapeptide libraries were synthesized as C-terminal amides because the exotic amino acids are more easily immobilized on the corresponding resin. The screening process of the libraries constructed from the set of optidiverse amino acids led to the tryptophan analog Bta in position 1 as the best inhibiting sublibrary (Fig. 5). After resynthesis of the Bta sublibraries, the amino acid at position 2 corresponding to the highest inhibition were Bta, Fpa, Clp and Cha from which Fpa was chosen. The next step selected Cha as the best residue in position 3. Finally, the last step of selection on a set of unique analogs H-Bta-Fpa-Cha-O4-NH2, identified the best inhibition for O-4 = Phe, Clp, Fpa, Bta and Cha. These five peptides were assayed for their IC50 value toward the enzyme and displayed values in the low micromolar range (4–27 μM) (Table IV).

For the biased pentapeptide library (not shown), tryptophan was used at position 1 (N terminus) in all the sublibraries, since analogs of tryptophan seem to bear structural features that are recognized by the enzyme. The next selection round gave 4-nitrophenylalanine at position 2. Then valine was selected for the position 3 among several possibilities, including Leu, Nip, and Trp. For the position 4, the selection was made among Ile, Nip, Phe, Trp, and Val, and isoleucine was chosen. The final library, a series of unique pentapeptides (Trp-Nip-Val-Ile-O4), gave among several options for the C terminus, O4 = Nal1, Nip, and Nle, which led to the highest inhibition at the lowest tested concentration (0.01 mg/ml). In all cases, selected peptides were purified and chemically analyzed, and the enzymatic tests were done in great detail (Table IV).

Finally, one of the best peptide inhibitors discovered, S 34461 (Trp-Nip-Val-Ile-Nal1), was submitted to an Ala scan, a Gly scan, and to a deletion of each amino acid in turn at each position (Table V). The deletion tetrapeptides showed decreased potencies by a factor of 10 (des-Trp, des-Nip, and des-Val) to 100 (des-Ile and des-Nal1) compared with the initial pentapeptide. The Ala scan showed that the N-terminal position was not crucial for the biological activity. Similarly, the Gly scan showed that the mutation of Trp to Gly (at the N terminus) did not modify the inhibitory potency of the peptide.

### Table III

| Compounds | Structure | Activity (%) of serotonin | Vmax (mol/min) | Km (μM) |
|-----------|-----------|---------------------------|---------------|---------|
| serotonin | indole    | 100                       | 1750 ± 115    | 83 ± 12.5 |
| tryptamine | indole    | 176                       | 880 ± 71      | 702 ± 25.3 |

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### Table IV

| Compound     | Structure                  | IC50 μM ± S.E. |
|--------------|----------------------------|----------------|
| S 34461      | Trp-Nip-Val-Ile-Nal1       | 4.3 ± 1.3      |
| S 34503      | Trp-Nip-Val-Ile-Nip        | 5.5 ± 0.2      |
| S 34504      | Trp-Nip-Val-Ile-Phe        | 10.9 ± 2.7     |
| S 34469      | Trp-Nip-Val-Ile-Nle        | 27.9 ± 2.1     |
| S 34425      | Trp-Nip-Val-Ile-Tyr        | 45.3 ± 20.4    |
| S 35116      | H-Bta-Fpa-Cha-Bta-NH2      | 4.2 ± 0.2      |
| S 35122      | H-Bta-Fpa-Cha-Cha-NH2      | 4.5 ± 0.1      |
| S 35117      | H-Bta-Fpa-Cha-Clp-NH2      | 4.1 ± 0.1      |
| S 35118      | H-Bta-Fpa-Cha-Fpa-NH2      | 7.6 ± 1.4      |
| S 35123      | H-Bta-Fpa-Cha-Phe-NH2      | 4.4 ± 0.1      |
| S 35124      | H-Bta-Fpa-Cha-Tyr-NH2      | 27.4 ± 0.3     |
| S 34381      | Bta-Nal1-Nle               | 159.0 ± 48.0   |
| S 34380      | Bta-Nal1-Leu               | 206.5 ± 29.5   |
| S 34528      | Bta-Nal1-Asp               | 212.0 ± 6.0    |
| S 34578      | Bta-Nal1-Met               | 175.5 ± 18.5   |

Peptides were resynthesized, purified, and thoroughly characterized. They were used as inhibitors in the standard assays at concentrations ranging from 10−3 to 10−7 M. Experiments were done two to four times, depending on the peptides, and results are given as means ± S.E.

### Table V

| Compound     | Structure                  | IC50 μM ± S.E. |
|--------------|----------------------------|----------------|
| S 34469      | Trp-Nip-Val-Ile-Nle        | 27.9 ± 2.1     |
| S 34425      | Trp-Nip-Val-Ile-Tyr        | 45.3 ± 20.4    |
| S 35123      | H-Bta-Fpa-Cha-Phe-NH2      | 4.4 ± 0.1      |
| S 35124      | H-Bta-Fpa-Cha-Tyr-NH2      | 27.4 ± 0.3     |
| S 34381      | Bta-Nal1-Nle               | 159.0 ± 48.0   |
| S 34380      | Bta-Nal1-Leu               | 206.5 ± 29.5   |
| S 34528      | Bta-Nal1-Asp               | 212.0 ± 6.0    |
| S 34578      | Bta-Nal1-Met               | 175.5 ± 18.5   |

For the biased pentapeptide library (not shown), tryptophan was used at position 1 (N terminus) in all the sublibraries, since analogs of tryptophan seem to bear structural features that are recognized by the enzyme. The next selection round gave 4-nitrophenylalanine at position 2. Then valine was selected for the position 3 among several possibilities, including Leu, Nip, and Trp. For the position 4, the selection was made among Ile, Nip, Phe, Trp, and Val, and isoleucine was chosen. The final library, a series of unique pentapeptides (Trp-Nip-Val-Ile-O4), gave among several options for the C terminus, O4 = Nal1, Nip, and Nle, which led to the highest inhibition at the lowest tested concentration (0.01 mg/ml). In all cases, selected peptides were purified and chemically analyzed, and the enzymatic tests were done in great detail (Table IV).

Finally, one of the best peptide inhibitors discovered, S 34461 (Trp-Nip-Val-Ile-Nal1), was submitted to an Ala scan, a Gly scan, and to a deletion of each amino acid in turn at each position (Table V). The deletion tetrapeptides showed decreased potencies by a factor of 10 (des-Trp, des-Nip, and des-Val) to 100 (des-Ile and des-Nal1) compared with the initial pentapeptide. The Ala scan showed that the N-terminal position was not crucial for the biological activity. Similarly, the Gly scan showed that the mutation of Trp to Gly (at the N terminus) did not modify the inhibitory potency of the peptide.
bars represent the variation to the individual data. The labels on the D.

Three rounds of deconvolution included screening and synthesis of respectivley, in the individual sublibraries.

were run as described under “Material and Methods” using the HPLC four different concentrations of serotonin were used. The real black bars A and 0.1 mg/ml (hatched bars concentrations of 1 mg/ml (N-

plotted according to Lineweaver-Burk, led to a series of e.g. and cosubstrate, by varying the concentrations of one compo-

method of enzymology for the study of two substrates enzymes, to the method described by Segel (38). According to the classical hAANAT. Therefore, the best inhibitors were tested according of the molecule in or in the vicinity of the active site of

should bring important information regarding the orientation recorded with the peptides and the bisubstrate inhibitor

FIG. 5. Deconvolution of a tetrapeptide library as source of human arylalkylamine N-acetyltransferase inhibitors. Assays were run as described under “Material and Methods” using the HPLC assay. Individual sublibraries were solubilized in 10% Me2SO at the concentrations of 1 mg/ml (hatched bars) and 0.1 mg/ml (black bars) (A and B) and 0.1 mg/ml (hatched bars) and 0.01 mg/ml (black bars) (C and D). Three rounds of deconvolution included screening and synthesis of the best candidate-containing set of sublibraries. All the assays were done twice and the data represented as the resulting mean. The error bars represent the variation to the individual data. The labels on the x axis represent the amino acid residue in the position O1, O2, O3, or O4, respectively, in the individual sublibraries.

compared with the parent peptide. Both scans showed that the other positions were important for the inhibitory potency, since the mutations at all other positions led to a 10-fold loss in potency. These results confirmed those obtained with the tetrapeptide library showing that shorter structures (tetrapeptide size) were as potent as pentapeptides to inhibit hAANAT.

Enzymatic Studies of Inhibitors—The nature of the inhibition recorded with the peptides and the bisubstrate inhibitor should bring important information regarding the orientation of the molecule in or in the vicinity of the active site of hAANAT. Therefore, the best inhibitors were tested according to the method described by Segel (38). According to the classical method of enzymology for the study of two substrates enzymes, the real \( K_m \) and \( V_{\text{max}} \) values were determined toward substrate and cosubstrate, by varying the concentrations of one compo-
nent (e.g., the substrate) at different concentrations of co-substrate. The sets of straight lines obtained when these data were plotted according to Lineweaver-Burk, led to a series of \( V_{\text{max}} \) (app), when those lines crossed the y axis. These \( 1/v \) axis intercepts were replotted against the 1/substrate concentration and led to a straight line intercepting the x axis at \( 1/K_m \) and having a slope equal to \( K_m/V_{\text{max}} \). Five concentrations of acetyl-CoA at four different concentrations of serotonin were used. The real \( K_m \) for serotonin, as expected, was 1.48 and 1.46 mM in the two independent series of measurements. For the acetyl-CoA, the real \( K_m \) values were 0.528 and 0.513 in the same two series of experiments.

By using the same approach, as also described by Segel (38), the influence of SD 715 and of the pentapeptide inhibitors (both at 4 different concentrations) was determined on the kinetics of hAANAT. Mixed inhibition was found for the pentapeptides, except for S 34461, suggesting interference in a region where both substrate and cosubstrate are present, such as in the vicinity of the catalytic site. Furthermore, the same experiments led to slightly different observations with S 34461 and SD 715 (Fig. 6), strongly suggesting interferences of these two inhibitors with the cosubstrate-binding site and to a lesser extent with the substrate-binding site. Overall, the \( K_m \) values recorded for these fve compounds ranged from 12 nM to 200 \( \mu \)M. Whenever comparison is possible and despite the different species (ovine versus human), the data reported by Khalil and Cole (51) match those reported herein for the bisubstrate inhibitor, SD 715.

Models of the Human AANAT Enzyme Complexed with the Peptide Inhibitors and Comparison with the Binding Model of the Bisubstrate Inhibitor, SD 715—The crystal structures of sheep AANAT enzyme in complex with the bisubstrate analog SD 715 (18) guided the molecular modeling investigations of the binding modes for peptide inhibitors. The key recognition contacts of the bisubstrate analog are shown in Fig. 7. Because S 34461 behaves like the bisubstrate analog, as a pure competitive inhibitor versus acetyl-CoA, the molecular modeling investiga-

tion was focused on the Trp-Nip-Val-Ile-Nal1 pentapeptide. In line with the central dogma of the arylalkylamine recognition by the enzyme, three possible alignments of the aromatic residues Trp1, Nip2, and Nal15, respectively, with the tryptamine fragment of the bisubstrate analog, were considered as plausible initial docking conformations. Two of these alignments, the Trp1 and Nip2 superimpositions, led to inverted polarity of the peptide backbone relative to the polarity of the triamide backbone of the bisubstrate analog and hence adopted retro binding modes. The Trp1 recognition hypothesis was not acceptable because it led to non-optimizable bad contacts for the residue Nip2 located between the \( \beta_2 \) and \( \beta_5 \) strands, which overlaps with residues at the bottom of the cavity. This would also explain the somewhat surprising re-

| Peptide analogs | IC50 \( \mu \text{M} \) |
|-----------------|------------------|
| Trp-Nip-Val-Ile-Nal1 | 4.3 ± 0.3 |
| Nip-Val-Ile-Nal1* | 40 ± 1.6 |
| Trp-Nip-Val-Ile-Nal1 | 40 ± 2.5 |
| Trp-Nip-Val-\( \beta \text{Nal1} \) | 44 ± 4.8 |
| Trp-Nip-Val-\( \beta \text{Nal1} \) | 213 ± 3.5 |
| Trp-Nip-Val-Ile-\( \beta \) | 348 ± 32.5 |
| Ala-Nip-Val-Ile-Nal1 | 3.9 ± 0.03 |
| Trp-Ala-Val-Ile-Nal1 | 25 ± 0.1 |
| Trp-Nip-Ala-Ile-Nal1 | 26.3 ± 1.2 |
| Trp-Nip-Ala-Nal1 | 20.6 ± 2.5 |
| Trp-Nip-Val-Ala-\( \beta \) | 41.6 ± 3.6 |
| Gly-Nip-Val-Ile-Nal1 | 5.4 ± 0.04 |
| Trp-Gly-Val-Ile-Nal1 | 38.2 ± 0.3 |
| Trp-Nip-Gly-Ile-Nal1 | 53.7 ± 1.7 |
| Trp-Nip-Val-Gly-Nal1 | 28.6 ± 3.6 |
| Trp-Nip-Val-Ile-Gly | 37.2 ± 3.9 |

* The symbol \( * \) indicates the deletion in the parent peptide.
results obtained with the Ala scan experiment. The Nip\textsuperscript{2} and NaI\textsuperscript{15} alignments provide both geometrically and sterically plausible docking complexes (e.g. no-cis peptide bonds and no bad contacts), which were energetically comparable (Fig. 7, B and C, respectively).

The two models revealed several specific binding interactions. According to the imposed constraints for the recognition at the arylalkylamine site, both models showed the grip of central aromatic ring (NaI\textsuperscript{15}, Fig. 7B, and Nip\textsuperscript{2}, Fig. 7C) inside the hydrophobic pocket and the grip of the central peptide bond NaI\textsuperscript{15}-Ile\textsuperscript{4} (Fig. 7B) or Trp\textsuperscript{1}-Nip\textsuperscript{2} (Fig. 7C) between the NH of Leu\textsuperscript{124} and the C=O of Met\textsuperscript{159}. Several non-imposed interactions were observed as follows. 1) For the NaI\textsuperscript{15} recognition hypothesis (Fig. 7B) the C-terminal carboxyl group is hydrogen-bound to the main chain NH of Glu\textsuperscript{161}; the NH of Ile\textsuperscript{4} is hydrogen-bound with C=O of Leu\textsuperscript{124}; the p-nitrophenyl group of Nip\textsuperscript{2} occupies the central part of the pantetheine cavity, and its negatively charged C terminus makes favorable interactions with the guanidinium group of Arg\textsuperscript{131}. 2) For the Nip\textsuperscript{2} recognition hypothesis (Fig. 7C), the Trp\textsuperscript{1} residue makes several hydrogen bonds as follows: its NH\textsubscript{3} is bound to N\textsuperscript{ε} of His\textsuperscript{122}; the indole NH is bound to N\textsuperscript{ε} of His\textsuperscript{122}, and the peptide C=O is bound the NH of Met\textsuperscript{159}; the NH of Nip\textsuperscript{2} makes a hydrogen bond to the C=O of His\textsuperscript{122}; the polar p-nitro group of Nip\textsuperscript{2} is located in an unfavorable manner inside the hydrophobic pocket and has contacts with Leu\textsuperscript{60}, Val\textsuperscript{62}, and Val\textsuperscript{183}; the naphthyl ring of NaI\textsuperscript{15} occupies the central part of the pantetheine cavity, and its negatively charged C terminus makes favorable interactions with the guanidinium group of Arg\textsuperscript{131}.

**DISCUSSION**

After hAANAT was cloned by Coon et al. (17), rather few biochemical data were collected from this enzyme of human origin, the ovine form having been described initially as structurally close to the human one. Crystallization attempts of the human enzyme have failed so far, due to the low solubility and strong instability of the protein. In contrast, crystallization and determination of the detailed x-ray structure of the sheep enzyme were reported (18–19).

Here, the purification of the human and of the ovine fused protein was done in parallel on the same construction. On the whole, the results for the ovine enzyme are similar to those reported in the literature (Table I). The electrophoretic profiles of purified and cleaved proteins from both origins were almost identical, suggesting that the differences in specific activities observed for the enzymes are not due to a difference in the purification level but rather to intrinsic catalytic capacities related to differences in amino acid sequences. Previous species to species comparisons for another transferase (UDP-glucuronosyltransferase) showed a limited catalytic activity of the human enzyme compared with rabbit, rat, mouse, pig, or...
Fig. 7. Stereoview of the active site of AANAT complexed with inhibitors. A, crystal structure of sheep AANAT in complex with the bisubstrate analog SD 715 (19). B and C, proposed models of the complex of human AANAT with the pentapeptide inhibitor S 34461 (Trp-Nip-Val-Ile-Nal1). B, Nal1 binding recognition hypothesis; C, Nip2 recognition hypothesis. Indicated are the residues that are within a 5-Å contact sphere of either ligand. The representation of the protein residues is limited to the side chain atoms with the exception of the two parallel β strand segments 122–126 (β5 strand) and 158–161 (β2 strand) which form the platform for the binding of the polyamide backbone of the ligands and the helical segment 131–137 which is involved in the binding of the pyrophosphate fragment of the bisubstrate analog. Residues mutated between the sheep and human enzyme within the binding site are as follows: Ser60 → Leu, Asn62 → Val, Ala123 → Val, Lys135 → Arg and Ser137 → Pro. These essential binding interactions of the bisubstrate analog are seen: 1) the tryptamine fragment has its indole bound in an hydrophobic cavity constituting of Phe56, Pro64, Met159, Leu183, and Phe188, and the amide group is inserted between the two parallel β strand segments 122–126 (β5 strand) and 158–161 (β2 strand) with two hydrogen bonds to the NH of Leu124 and the C=O of Met159, 2) the pantetheine fragment is positioned on the platform of the β5 strand segment His122, Val126 by two hydrogen bonds between the C=O of Leu124 with the NH of Phe188 and the NH of Val126 with the C=O of PO10 (pantetheine oxygen-10), hereby mimicking a parallel β sheet; 3) the 3P-ADP fragment is anchored by its pyrophosphate group inside the backbone groove of the N-terminal segment (residues 131–137) of the helix α5.

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guinea pig (61). A surprising finding was the “poor” $K_m$ values of hAANAT toward its natural substrates (i.e. in the millimolar range for serotonin and tryptamine), with the sole exception of PEA, while millimolar $K_m$ values toward acetyl-CoA had also been recorded in other species (33). However, $K_m$ values in the same range are not rare for other transferases toward their respective substrates (57–59). For the AANAT of other origin, various levels of $K_m$ for serotonin have been reported in sheep, rat, and even in fishes as follows: 170 (17), 20 (29) and 85 $\mu$M (15) for sheep, 2000 $\mu$M (15) for rat, 20 $\mu$M for pike (62) but 1400 $\mu$M for zebrafish (63). Furthermore, when the substrate was tryptamine instead of serotonin, $K_m$ values ranged from 1540 $\mu$M in human ovary (23), 50 (29), 260 (51) or 110 $\mu$M (15) in sheep, 3500 $\mu$M in rat to 2190 $\mu$M (33) in rabbit lens. The kinetic data recorded in sheep were checked versus the purified enzyme obtained from animals sacrificed under particularly strict (dark) conditions, since the AANAT undergoes to a very rapid catabolism (16) after light exposition with e.g. $-8.5$ min half-life in the rat (64). Thus, a similar experience on human post-mortem pineal gland-derived material is impossible to perform.

Therefore, in order to assess our kinetic data, we compared our results with those obtained under identical conditions from another animal, such as the sheep experiments conducted in two different laboratories on cloned material (17, 30). The data we obtained on purified fused protein (GST-oAANAT) are similar to those previously published (Table I) for both serotonin and tryptamine substrates, strongly suggesting, although indirectly, the validity of our methodology and therefore the relatively poor affinity of AANAT for its “natural” substrate.

Concerning the main contaminant of the GST-AANAT preparations, the bacterial chaperone protein, Hickman et al. (18), separated it from the oAANAT using gel filtration, which made possible the crystallization of the ovine transferase. We found a way to minimize the chaperone/hAANAT ratio in the purification process, which seems to reduce the strong interaction between these proteins.

The kinetic behavior of the sheep AANAT has been described by Cole’s group in recent publications (30, 31, 51, 65). Our data gathered in Table I on the sheep enzyme showed only minor differences. The relative potencies of the various substrates in human and in ovine were quite different from one species to the other (Table I). The main discrepancies were the behavior of PEA which was not a better substrate in sheep and rat than tryptamine or serotonin, while it was by far better in human (Table II), and the poor potency as substrates of both tryptamine and serotonin, when compared with the sheep (10 times less potent in human than in sheep). Whether this observation is of any consequence for the development of potent inhibitors remains to be documented, but this is a clear difference that must be kept in mind whenever structure-activity relationship are investigated. Among the amino acid differences of the two species enzymes, the loss of a serine and asparagine (Ser for Leu and Asn for Val) in the catalytic domain should render this area far more hydrophobic in the human enzyme, thus suggesting an explanation for the difference in affinities ($K_m$) between serotonin and tryptamine, since the latter lacks the hydroxyl group in the position 5.

AANAT has a fairly high specificity in terms of the restricted number of cosubstrates discovered so far. Indeed, only acetyl- and propionyl-CoA were able to act as a catalytic partners for the transfer reaction.

Combinatorial libraries were used in the past years to find enzyme inhibitors (see complete reviews in Refs. 66 and 67). This body of techniques is clearly an essential tool for the research of pharmacological leads. The most impressive reports include those of Wallace et al. (68) on S-farnesyltransferase inhibitors and of Songyang et al. (69, 70) on SH2 protein/protein interactions using one or two classical types of libraries. The present work presents a series of libraries in which the size and the chemical nature of the building blocks widely varied, giving rise to a large panel of structure-activity relationships for the final products obtained. A constant striking feature of the peptide leads found was their hydrophobic nature as seen, for example, from the structure of S 34461, which contains not less than three aromatic and two aliphatic non-charged residues.

The data obtained with the peptide inhibitors also indicate that a minimal length is required for efficient interaction, since the tripeptides ($M_r = 330$) were poor inhibitors compared with the tetrapeptides ($M_r = 440$) and pentapeptides ($M_r = 550$). Furthermore, the synthesized analogs of the S 34461 that were shorter than the parent compound showed a general decrease of their inhibitory potency. The library tetrapeptides S 35116 and S 35122 (Table IV) and those issued from S 34461 (Table V) may be invaluable leads for chemical optimization toward non-peptide inhibitors of the hAANAT.

The immediate goal of the proposed three-dimensional models of the enzyme-peptide inhibitor complex obtained from molecular modeling was to suggest chemical modifications of the inhibitors for design purposes. For the Nal15 recognition mode, for instance, the modification of the C terminus of Nal15, elimination or substitution by non-charged hydrogen bond acceptor groups, should lead to improvement. Both binding hypotheses suggest in comparison with the bisubstrate analog, two additional recognition sites, Arg121 and Glu161, which are accessible inside the binding site and potentially useful for design purposes. Furthermore, the mutations within the binding site (sheep to human), especially the mutations Ser60 → Leu and Asn62 → Val which are in proximity of the indole-5 position of the tryptamide fragment of the bisubstrate analog, make the human aryalkylamine site significantly more hydrophobic than in the sheep enzyme.

More fundamentally, the three-dimensional models of the inhibitor enzyme complexes allow one to understand the apparent type of inhibition. The competitive inhibition of the pentapeptide inhibitors versus the acetyl-CoA cosubstrate is appropriately rationalized in both binding models because of the partial occupation of the acetyl-CoA site by the inhibitor. The same holds for the bisubstrate analog that has the potential for total occupation of the acetyl-CoA site. The non-competitive nature of the bisubstrate analog versus the serotonin cosubstrate, i.e. the apparent reduction of $V_{max}$ for increasing inhibitor concentrations, might equally be rationalized in terms of structural models by accepting the formation of a ternary complex involving the binding of serotonin at the aryalkylamine site and the binding of the acetyl-CoA part of the bisubstrate analog at the acetyl-CoA cosubstrate site, the tryptamide part of the inhibitor would hence be bound elsewhere. Because such a complex would result in a reduction of the enzyme concentration it would affect $V_{max}$. Similar considerations might apply for peptide inhibitors.

In conclusion, the experiments reported here demonstrate that the human form of AANAT can be expressed from its clone in a bacterial stem and partially purified in a reproducible way and in a short number of steps to a protein preparation with a specific activity comparable to that of the ovine enzyme. The human form recognizes the classical substrates of AANAT, such as serotonin and tryptamine, and acetylates more easily PEA than does the ovine AANAT. This is apparently due to structural differences in its active site, including the substitution of Ser60 and Asn62 to Leu and Val, respectively, which
allows the hydrophobic core of PEA to be accommodated in the human active site, as shown by molecular modeling. AANAT is also shown in the present report to accommodate synthetic arylyethlamines with similar potency than for some natural amines. In addition, the herein discovered tetrapeptide sequences, such as those derived from S 34461, could constitute useful leads for the development of potent non-peptide enzyme inhibitors.

Acknowledgments—We are deeply indebted to Drs. D. C. Klein, S. L. Coon, and M. A. A. Namboodiri (National Institutes of Health, Bethesda, MD) for providing the ovine and human GST-AANAT constructs, experimental guidelines, and helpful discussions. We are also grateful to Dr. D. C. Klein for comments on the present manuscript and to Nicole Taimiot and Solange Huet for expert help in editing the manuscript.

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J. Biol. Chem. 2000, 275:8794-8805.
doi: 10.1074/jbc.275.12.8794

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Additions and Corrections

Vol. 275 (2000) 8794–8805

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Throughout the text, all of the $V_{\text{max}}$ units read “$\mu$mol/min/mg protein,” whereas they should read “nmol/min/mg protein.” This mistake is especially evident in the following occurrences.

**Page 8797, right column:** The next to last sentence in the last paragraph should read: “Comparison of the data obtained with this HPLC assay with those generated with the extraction assay showed similar activities (not shown) agreeing at least within a factor 2: 1.33 and 1.66 nmol/min/mg protein for activities measured with the fused hAANAT and 16.6 and 33.3 nmol/min/mg protein for the pure hAANAT.”

**Page 8799, left column:** The 11th line from the bottom of the page should read: “enzyme with a $V_{\text{max}}$ of 438 ± 27 nmol/min/mg of protein.”

**Page 8799, right column:** The 10th through 14th lines should read: “Nevertheless, none of the alternate synthetic substrates had better rate constants than PEA (~4000 nmol/min/mg protein) or tryptamine (~800 nmol/min/mg protein), whereas for serotonin, it was comparable to the aryl bicyclic compounds (~200–300 nmol/min/mg protein).”

This correction should also be made in the unit headings of Tables II and III (pages 8799 and 8800, respectively).

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.