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The Structural Basis of Ordered Substrate Binding by Serotonin N-Acetyltransferase: Enzyme Complex at 1.8 Å Resolution with a Bisubstrate Analog

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Summary

Serotonin N-acetyltransferase, a member of the GNAT acetyltransferase superfamily, is the penultimate enzyme in the conversion of serotonin to melatonin, the circadian neurohormone. Comparison of the structures of the substrate-free enzyme and the complex with a bisubstrate analog, coenzyme A-S-acetyltryptamine, demonstrates that acetyl coenzyme A (AcCoA) binding is accompanied by a large conformational change that in turn leads to the formation of the serotonin-binding site. The structure of the complex also provides insight into how the enzyme may facilitate acetyl transfer. A water-filled channel leading from the active site to the surface provides a pathway for proton removal following amine deprotonation. Furthermore, structural and mutagenesis results indicate an important role for Tyr-168 in catalysis.

Introduction

Many aspects of metabolism display circadian cycles that optimize function and survival by allowing organisms to anticipate and coordinate changes in the 24 hr environmental lighting cycle. Such rhythms are seen in nearly all life forms and appear to be driven by molecular clocks composed of oscillating biochemical feedback systems involving transcription factors (reviewed in Reppert, 1998; Dunlap, 1999). An important output signal of biological clocks in vertebrates is melatonin, which appears to provide a highly reliable indication of day and night; in all vertebrates, circulating melatonin is ~10-fold higher at night. Melatonin is thought to function both as a downstream signal and also as a feedback signal for the biological clock. For example, one of the most consistently high levels of melatonin receptors in mammals is found in the suprachiasmatic nucleus—the "mind's clock" (Klein et al., 1991). It is thought that actions of melatonin on this neuronal structure may be the basis of effects that melatonin has on jet lag (Wirz-Justice, 1995).

Circulating melatonin is produced by the pineal gland, where it is synthesized by the sequential action of two enzymes (reviewed in Klein et al., 1997). Serotonin-N-acetyltransferase (arylalkylamine-N-acetyltransferase [AA-NAT]) first catalyzes the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to the primary amine of serotonin; the product of this reaction, N-acetylserotonin, is then methylated at the 5-hydroxy position by hydroxyindole-O-methyltransferase (HIOMT) to produce melatonin (Figure 1). The regulation of the large daily changes and light-induced changes in melatonin levels appears to be at the level of AANAT activity, since its levels also vary in parallel with those of melatonin. For example, in some species the night/day differences in melatonin, AANAT activity, and protein are 10- to 100-fold; light exposure at night causes these to rapidly drop with a halving time of ~3.5 min (Klein and Weller, 1972; Gastel et al., 1998).

We recently described the three-dimensional structure of AANAT, determined at 2.5 Å resolution (Hickman et al., 1999). AANAT is a member of a large superfamily of proteins, referred to alternatively as the motif A/B or the GCN-5-related N-acetyltransferase (or GNAT) family, whose members have been identified on the basis of four conserved sequence motifs (Neuwald and Landsman, 1997). The function of these enzymes is to transfer the acetyl group of AcCoA to a wide variety of substrates, ranging from small molecules to large nuclear protein complexes. AANAT consists of a highly curved β sheet flanked by several α helices, which is pulled apart toward its center between two parallel strands. This results in a "V" or a wedge leading into the interior of the protein. Three loops converge over the β sheet, creating a funnel lined with hydrophobic residues, which were suggested to form the serotonin-binding site. The structures of two other enzymes in the GNAT family have also been determined (reviewed in Modis and Wierenga, 1998), those of the yeast histone acetyltransferase Hat1 (Dutnall et al., 1998) and a eubacterial aminoglycoside 3-N-acetyltransferase, AAT (Wolf et al., 1998). The structure of Hat1 was determined in the presence of AcCoA, while that of AAT was solved with bound CoA. Comparison of the structures reveals that they possess a common central fold, and the "V" in the center of the β sheet forms the AcCoA-binding site into which the pantetheine group of coenzyme A is inserted. By analogy, AcCoA was predicted to bind to AANAT in a similar manner, but it was apparent that for binding to occur, there would have to be movement of some of the residues in one of the loops (Hickman et al., 1999). Despite the similarity in their AcCoA-binding regions, the three structures diverge significantly in other regions presumed to be involved in binding the substrate to be acetylated.

We now present a high-resolution structure of AANAT in which a bisubstrate analog is bound at the enzyme active site. The analog, synthesized by covalently linking an N-acetylated substrate, tryptamine, to CoA, mimics

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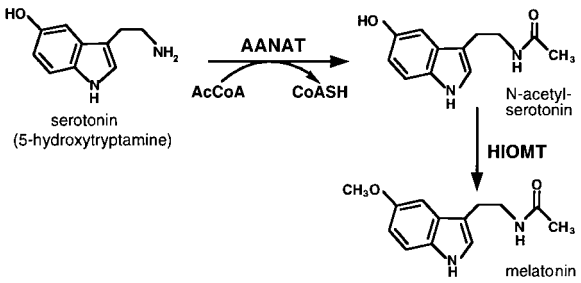

Figure 1. Biochemical Pathway for the Synthesis of Melatonin

Melatonin is synthesized from serotonin by the sequential action of serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase [AANAT]) and hydroxyindole-O-methyltransferase (HIOMT).

the structure of the presumed intermediate formed during catalysis and was first described and characterized by Khalil and Cole (1998). The structure of the complex with the bisubstrate analog allows us to examine a number of issues relating to catalysis, including the nature of substrate binding and the mechanism of enzyme action. It also serves as a starting point for the rational development of tightly binding inhibitors that may be of pharmacological significance for the treatment of clinical problems in which melatonin has been implicated (such as sleep regulation and jet lag) and for the treatment of diseases in which serotonin plays a role, such as depression or obesity (for recent reviews, see Bonhomme and Esposito, 1998; Kordik and Reitz, 1999). Furthermore, the structure may be valuable in understanding the ways in which the important neurotransmitter serotonin may be bound to other proteins of physiological interest.

Results and Discussion

Coenzyme A-S-Acetyltryptamine Bisubstrate Analog

The bisubstrate analog used in this study was shown by Khalil and Cole (1998) to be a linear competitive inhibitor against AcCoA, and a noncompetitive inhibitor versus tryptamine with an IC_{50} of ~150 nM when assayed using full-length sheep AANAT. In the present study, it was determined that this compound is also a potent inhibitor of a truncated form of sheep AANAT consisting of residues 28–201 ($AANAT_{28–201}$).

Complex Formation and Structure Determination

We previously described the structure of uncomplexed $AANAT_{28–201}$, which readily crystallized under conditions of low ionic strength (Hickman et al., 1999). This construct, which is fully active, was chosen for study, since the full-length protein was susceptible to proteolysis at several sites at its N terminus, suggesting this region might be disordered; furthermore, the N-terminal sequence (residues 1–27) is not well conserved among AANATs from various species (Klein et al., 1997). In the current study, formation of a 1:1 complex between the bisubstrate analog and $AANAT_{28–201}$ (subsequently called AANAT) was verified by analytical gel filtration, and crystals that diffract to high resolution were obtained by the sparse matrix sampling method (Jancarik and Kim, 1991) followed by several cycles of macroseeding (see Experimental Procedures). Mass spectrometry measurements on dissolved crystals confirmed the presence of intact CoA-S-acetyltryptamine. The structure of the complex was solved using molecular replacement (see Experimental Procedures and Table 1 for details). The current model includes all residues except the first four N-terminal and the final six C-terminal residues.

Table 1. Data Collection and Refinement Statistics

Data Collection	
Resolution (Å)	30–1.8
Total reflections (N)	96,003
Unique reflections (N)	15,197
Completeness (%) (for $I/\sigma I > 0.0$)	97.0
$I/\sigma I$	18.0
R_{sym}	0.041
Highest shell (between 1.85–1.80 Å)	
Completeness (%)	97.4
$I/\sigma I$	10.1
R_{sym}	0.121
Refinement	
Resolution (Å)	30–1.8
Atoms (N)	1,640
Reflections $F > 2\sigma(F)$	14,963
R factor (%)	18.1
R_{free} (%)	23.1
Rms bond lengths (Å)	0.006
Rms bond angles (°)	1.397

$R_{sym} = \Sigma|I - <I>|/\Sigma<I>$.

R factor $= \Sigma\ |FP_o - FP_c|/\Sigma|FP_o|$.

R_{free} is computed using 5% of the total reflections selected randomly and never used in refinement.

The Structure of the CoA-S-Acetyltryptamine–AANAT Complex

The electron density map shows clear and interpretable electron density in the enzyme active site corresponding to the bound bisubstrate analog. The analog is held in a conformation that is approximately "S"-shaped (Figure 2), a binding mode enforced in part by the protrusion of Phe-56 into the enzyme active site. As predicted from the structure of the uncomplexed enzyme, the pantetheine group is aligned approximately parallel to strand β5 of the central β sheet, held in place by a number of hydrogen bonding contacts, involving either main chain atoms or water-mediated hydrogen bonds; these and other interactions between CoA-S-acetyltryptamine and AANAT are detailed schematically in Figure 3. The positioning of the pantetheine moiety and the α- and β-phosphates of the ADP group is essentially identical to that seen in the complexes between AcCoA and Hat1 (Dutnall et al., 1998) and CoA and AAT (Wolf et al., 1998). The remainder of the ADP moiety, beyond the α- and β-phosphates, is more mobile, corresponding to the lack of specific interactions with the enzyme, a feature of coenzyme A binding also observed for the other structurally characterized members of the GNAT superfamily. We are certain that the observed position of the CoA portion of CoA-S-acetyltryptamine represents the authentic binding site for the AcCoA substrate, since comparison of the analog-bound form of AANAT and the AcCoA-bound form of Hat1 indicates that essentially

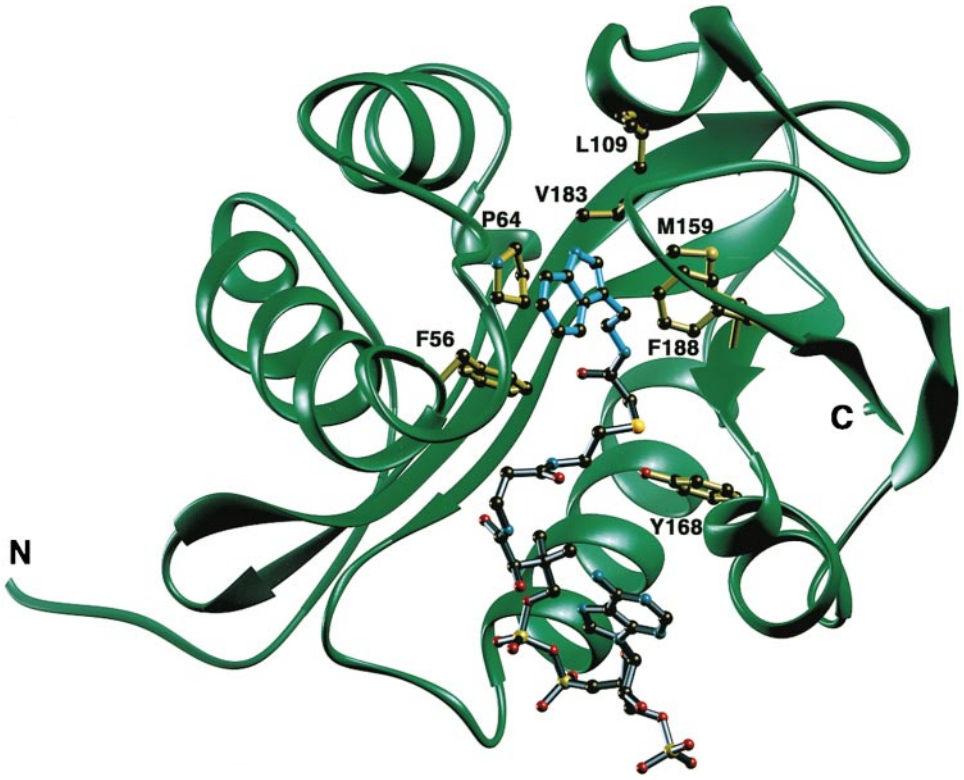

Figure 2. AANAT–Bisubstrate Analog Complex

The tryptamine moiety is shown in light blue, and the atoms of the bisubstrate analog are colored as follows: oxygen (red), nitrogen (dark blue), sulfur (yellow), phosphorus (gold). For clarity, not all of the side chains that line the hydrophobic serotonin-binding site are shown. The figure was made with RIBBONS (Carson, 1991).

identical interactions are involved in binding the pantetheine moiety and the phosphate groups; furthermore, the two structures can be aligned over 78 $C\alpha$ atoms with a root-mean-square (rms) deviation of 1.6 Å.

The sulfur atom of the pantetheine group is located within hydrogen bonding distance of residue Tyr-168 (3.1 Å), as previously predicted based on a structure-based alignment between AANAT and Hat1 (Hickman et al., 1999); in the alignment of the bisubstrate analog-bound form of AANAT and the AcCoA-bound form of Hat1, the sulfur atom positions differ only by 0.61 Å, which is within the expected error. The alkylamine nitrogen of the tryptamine moiety is linked to the pantetheine sulfur by two intervening carbon atoms, which represent the transferred acetyl group (-S-CH_2(CO)-N- as shown in Figure 3). The positions of the atoms in this important region of the bisubstrate analog are established by three hydrogen bonding contacts: those from Tyr-168 to the pantetheine sulfur, from the main-chain amide of Leu-124 to the carbonyl oxygen of the transferred acetyl group, and from the tryptamine amine nitrogen to the main-chain carbonyl oxygen of Met-159.

The indole ring of the tryptamine group is held in place through extensive hydrophobic contacts with six residues (Phe-56, Pro-64, Met-159, Val-183, Leu-186, and Phe-188), which converge to form a pocket that will be referred to here as the serotonin-binding site. This terminology recognizes the primary physiological substrate of the enzyme; however, it should be noted that AANAT acetylates a wide range of arylalkylamines. Also contributing to this serotonin-binding site are residues Leu-109 and Ile-181. In AANATs from other species, these eight residues are either invariant (in the cases of Phe-56, Pro-64, Leu-186, and Phe-188) or conservatively substituted with other hydrophobic residues (Klein et al., 1997). Thus, the main forces holding arylalkylamine substrates in place appear to be van der Waals interactions and hydrophobic packing. As shown in Figure 2, the indole ring of the bisubstrate analog is sandwiched between Pro-64 and Phe-188, a stacking interaction that likely stabilizes the entire serotonin-binding site.

Although the hydrophobic packing interactions cannot be considered to be specific, it seems reasonable to assume that the observed location of the bound tryptamine moiety represents the authentic binding site for serotonin. The hydrophobic pocket is of the correct shape and size to accommodate an arylalkylamine substrate, it is solvent accessible, thereby providing a means for the substrate to enter and the N-acetylated product to leave, and it leads directly to the acetyl group to be transferred. The only other structural information on the binding of serotonin to a protein is provided by the crystal structure of sepiapterin reductase bound to a competitive inhibitor, N-acetylserotonin (Auerbach et al., 1997). In this case, N-acetylserotonin appears to fortuitously form some of the same binding contacts with the protein as sepiapterin; its binding mode may

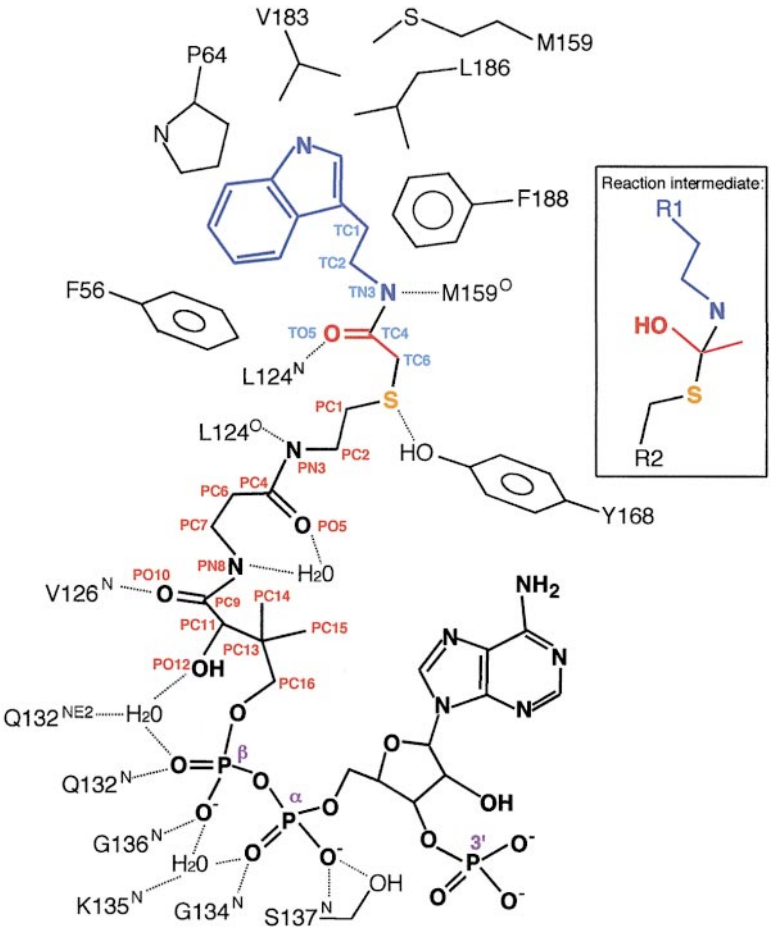

Figure 3. Schematic Diagram Showing the Bisubstrate Analog–Protein Contacts

Superscripted N's and O's associated with residue numbers indicate interactions with main-chain amine and carbonyl oxygens, respectively. The dashed lines indicate hydrogen bonds, and the cluster of amino acids near the tryptamine moiety indicate residues within van der Waals distance of the indole ring. For comparison, the box on the right-hand side shows the presumed tetravalent intermediate in the acetyl transfer reaction.

not be completely relevant for an understanding of those of authentic serotonin-binding proteins.

A hydroxy group at the 5-position of the indole ring, representing the only difference between serotonin and tryptamine, would be located within hydrogen bonding distance of Ser-60. It is unlikely that this interaction confers significant binding energy to the serotonin substrate, since in full-length sheep AANAT, tryptamine and serotonin have similar K_M values (Coon et al., 1995).

Comparison of the Structures of the Bisubstrate Analog–AANAT Complex and the Uncomplexed Form

The overall structures of the uncomplexed and CoA-S-acetyltryptamine-bound forms of AANAT are generally similar and can be aligned with an overall rms deviation in 138 Cα positions of 1.2 Å. The major structural difference between the two forms of AANAT occurs in the region previously designated as loop 1, which extends approximately from residues 40 to 80. In the original structure of the uncomplexed enzyme, we identified three protein loops converging over the enzyme active site to form a funnel leading toward the interior of the protein (Hickman et al., 1999). In the protein–CoA-S-acetyltryptamine complex, loop 1 has undergone a dramatic conformational change; a comparison of the two structures is shown in Figure 4. In uncomplexed AANAT (Figure 4A), two α helices mark the approximate beginning and end of loop 1 as it meanders across the face of the β sheet. This loop was clearly the most mobile region of the protein, although it showed up as continuous electron density in the original experimental (model bias-free) maps, and it had been suggested that part of it might have to move to allow the enzyme to accommodate AcCoA. Indeed, in the presence of the bisubstrate analog (Figure 4B), helix α1 in loop 1 is far more extensive: helix α1, which in the uncomplexed enzyme was observed to consist of residues 42–50, is unwound by two residues at its N terminus but now extends to residue 61 at its C-terminal end (with a short break in the middle, discussed below). The extension of this helix renders this region more rigid and results in a conformation that is more similar to those of AAT and Hat1 complexed to coenzyme A, both of which exhibit greater helical content in this region than does the uncomplexed form of AANAT. The similarity of the conformation in this region of the protein among the three structurally characterized members of the GNAT superfamily argues that the observed conformational change in loop 1 of AANAT is due to binding to the CoA portion of the bisubstrate analog, since neither of the other two structures contains the substrate to be acetylated.

There are two major structural consequences of the conformational change in loop 1. The first is a global

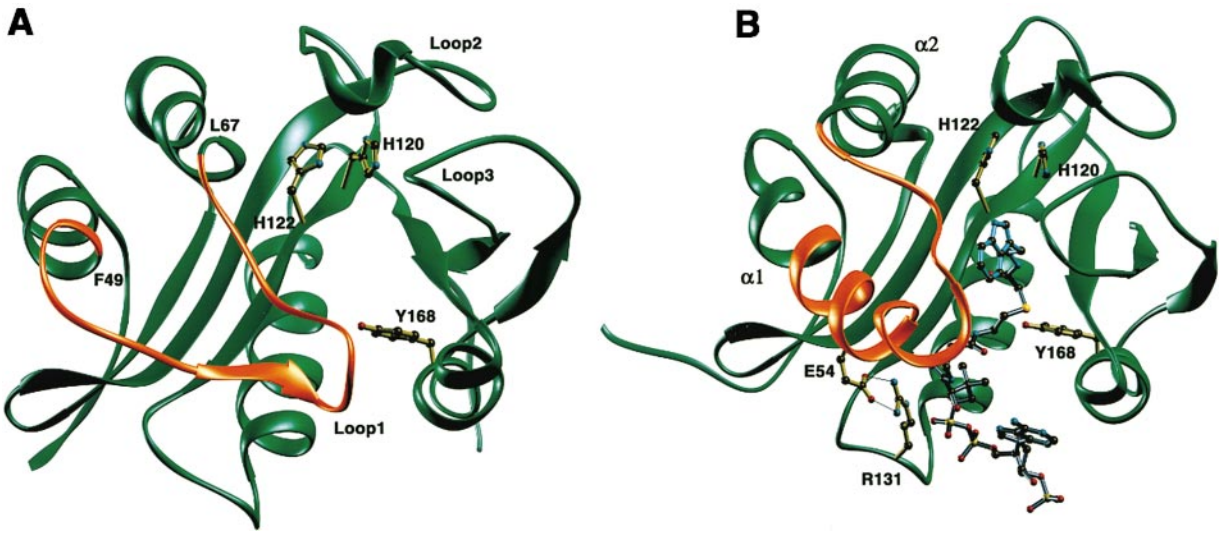

Figure 4. Comparison of Uncomplexed AANAT and the Bisubstrate Analog Complex

RIBBONS (Carson, 1991) representations of the structure of the uncomplexed enzyme showing the three loops converging over the active site (A) and the structure of the protein when bound to the bisubstrate analog (B). The segment of the polypeptide chain that rearranges upon acetyl coenyzme A binding (Phe-49 to Leu-67) is shown in gold. Note in (B) the extension of helix α1, the movement of the loop residues up and out of the pantetheine-binding site, and the loss of strand β2.

movement and restructuring of approximately 20 residues, around the pivot points represented by residues Phe-49 and Leu-67, up and out of the pantetheine-binding site. The two residues that move the furthest are Ile-57 and Ser-58, which are displaced by 12.5 and 13.6 Å, respectively. The loop conformation observed in the complex is stabilized, in part, by a salt bridge between the conserved residues Glu-54 and Arg-131; the salt bridge also causes a break in helix α1 around residues Ala-55 and Phe-56 (see Figures 2 and 4B). The movement of the loop leads to the loss of a short β strand (strand β2), consisting of three residues, Ala-55, Phe-56, and Ile-57, which occupies the AcCoA-binding site in the uncomplexed enzyme (see Figure 4A). In the absence of substrates, strand β2 forms hydrogen bonds to residues in strand β5: the main-chain carbonyl of Ala-55 to the main-chain amide of Val-126, and the main-chain amide of Ile-57 to the main-chain carbonyl of Leu-124. In the bisubstrate analog complex, the main-chain contacts involving the two residues on strand β5, Val-126 and Leu-124, are now directly to atoms PO10 and PN3 of the pantetheine moiety (see Figure 3). It seems likely that loop 1 is capable of assuming a number of conformations, and in one of these—that observed in the uncomplexed enzyme—part of it sits in the AcCoA-binding site, thereby hindering its binding. This is consistent with the relatively high K_M in the 0.2–0.3 mM range observed for this substrate (De Angelis et al., 1998).

The second effect of the conformation change in loop 1 is to form—or complete—the binding site for serotonin. As mentioned earlier, the resulting binding site positions the indole ring in a hydrophobic pocket formed by a number of converging residues. We had previously suspected that several of these residues might be involved in substrate binding, based on their positions in the uncomplexed protein (Hickman et al., 1999). The structure of the bisubstrate analog complex indicates that two additional residues, Phe-56 and Pro-64, contribute to the formation of the tight cavity that holds the indole ring in place, since the reorganization of loop 1 brings these residues close to the other hydrophobic residues that line the serotonin-binding site. Therefore, it appears that the binding site for serotonin is formed—or induced—only upon binding of AcCoA, with the associated change in the conformation of loop 1. This unveiling of a "cryptic binding site" for serotonin upon AcCoA binding provides a satisfying structural explanation for the kinetic results of De Angelis et al. (1998), which indicated an ordered binding mechanism for AANAT requiring binding of AcCoA prior to arylalkylamine substrates.

The bisubstrate analog used in these studies serves as a chemically stable mimic of the intermediate that might be expected during catalysis by AANAT: the analog contains the same number of carbon atoms as the presumed tetravalent intermediate, although their arrangement is slightly different (the comparison is shown in Figure 3), and the analog is not precisely one of the structures expected along the reaction pathway. Despite the subtle differences, it is reasonable to examine the issue of how CoA-S-acetyltryptamine binding provides insight into the catalytic mechanism of AANAT.

Implications for Catalysis

Since primary alkylamines such as serotonin and tryptamine have pKa values close to 10 (Merck Index, 1976), it is clear that amine deprotonation must precede acetyl group transfer (Figure 1). Indeed, Cole and coworkers showed that AANAT preferentially binds the protonated form of the substrate (Khalil et al., 1998), and several lines of evidence have implicated histidine residues in the deprotonation. Kinetic analyses using poor substrates such as N^{ω}-methyltryptamine suggested the involvement of an ionizable group with a pKa of ~7

Table 2. Effect of Site-Specific Mutations on the Activity of GST-AANAT Fusion Proteins

Fusion Protein	V_{max} (nmol/hr/ng Protein)	K_M (mM)
GST-AANAT$_{28–201}$		
Wild type	2.1	0.13
H120Q	2.2	0.85
H122Q	2.3	3.1
Y168F	0.07	3.5

(Khalil et al., 1998); in addition, the structure of the uncomplexed protein indicated that the hydrophobic funnel led directly toward two conserved histidines, His-120 and His-122 (Hickman et al., 1999). If the protein–CoA-S-acetyltryptamine complex accurately reflects the position of the amine nitrogen of the substrate (atom TN3) prior to catalysis, this places it 7.5 Å from the NE2 nitrogen of His-120 and 8.7 Å from ND1 of His-122, too far for these residues to be directly involved in deprotonation. However, an indirect role for the histidine residues in catalysis is suggested by the results of site-directed mutagenesis studies with sheep AANAT$_{28–201}$ (Table 2), which indicate that mutation of either His-120 or His-122 to Gln causes an increase in the K_M for tryptamine, without significantly altering the apparent V_{max}. (In contrast, a different result was obtained for full-length human AANAT in which His-120→Gln and His-122→Gln mutations reduced activity >90% under standard assay conditions [M. A. A. N. and D. C. K., unpublished data]. Since it is unlikely that the catalytic mechanisms differ fundamentally between species, this may reflect an even greater increase in K_M values in the case of the human AANAT mutations.)

Instead of supporting a direct catalytic role for the conserved histidines, the structure of the CoA-S-acetyltryptamine complex instead reveals a remarkable network of clearly defined water molecules running between the tryptamine moiety and the histidine residues, as shown in Figure 5A. A "proton wire" consisting of at least eight hydrogen-bonded water molecules extends away from the alkylamine nitrogen, TN3, connecting it to both histidines, the indole nitrogen, and out of the enzyme active site to the protein surface. This local pocket of water molecules could serve as a sink for the exchangeable alkylamine proton or a conduit to ferry the charge away from the substrates, thereby facilitating nucleophilic attack on AcCoA; such proton wires have been previously observed in other enzymes (Meyer, 1992) and are the subject of much current investigation (Pomès and Roux, 1995; Kohen and Klinman, 1998; Marx et al., 1999). The proximity of the strictly conserved histidine residues to this "proton wire" suggests that proton removal from the active site could also involve these imidazole groups.

The structure of the complex also allows us to deduce

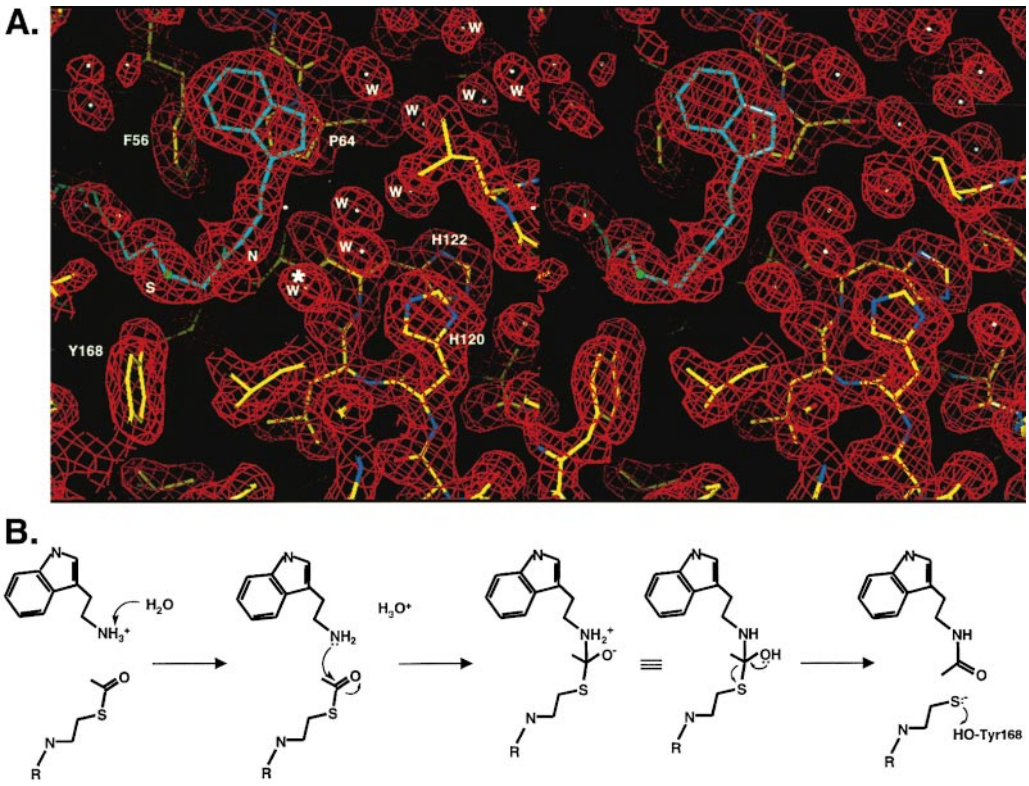

Figure 5. The Enzyme Active Site and Proposed Reaction Pathway

(A) 2Fo-Fc electron density map in stereo contoured at 1σ, showing electron density associated with the bisubstrate analog, and the binding pocket surrounding the tryptamine moiety (shown in blue). A water-filled channel (or "proton wire") links the tryptamine alkylamine and these histidine residues to bulk solvent; nine of the water molecules are labeled "W," and the one closest to the alkylamine nitrogen is labeled with an asterisk.

(B) Schematic of the reaction pathway for acetyl transfer from AcCoA to an arylalkylamine substrate.

the functional role in catalysis of the one-residue β bulge, previously noted close to the active site in strand β5 of the uncomplexed enzyme (Hickman et al., 1999). This β bulge places one residue out of register in relation to the repeating hydrogen bonding pattern of an antiparallel β sheet, and its effect is to point two consecutive main-chain carbonyl groups in the same direction. The main-chain carbonyls of Ile-121 and His-122 form hydrogen bonds to two of the water molecules of the proton wire, including the one located nearest to the alkylamine nitrogen (labeled with an asterisk in Figure 5A). This water molecule, which is also the most deeply located in the pocket, is coordinated by three hydrogen bonds and is 3.4 Å away from the alkylamine nitrogen, in an appropriate position to accept a proton. This intricate arrangement of water molecules ensures that, upon amine deprotonation, the proton can be conducted away from the active site. Contributing to the formation of the β bulge is the previously observed string of parallel-stacked aromatic residues leading from His-72, Phe-73, His-122, to His-120 (Hickman et al., 1999).

The design of the bisubstrate analog is such that the structure resembles that in which the acetyl group has already been transferred to the alkylamine nitrogen. In the CoA-S-acetyltryptamine complex with AANAT, the acetyl carbonyl oxygen is hydrogen bonded to the main-chain amide nitrogen of Leu-124. This residue provides a second hydrogen bond, via its main-chain carbonyl oxygen, to the most proximal pantetheine nitrogen (PN3 in Figure 3). The analogous residue in the AcCoA–Hat1 complex is Phe-220, and the main-chain interactions of Phe-220 of Hat1 with AcCoA mirror those of Leu-124 to the bound bisubstrate analog. Thus, the same interactions holding the relative positions of the acetyl and pantetheine groups of AcCoA bound to Hat1 are reiterated in the bisubstrate analog–AANAT structure. It is likely, therefore, that the role of Leu-124 of AANAT is to enforce the appropriate stereochemistry for the tetravalent intermediate by coordinating the carbonyl oxgyen of the acetyl group, both prior to and after transfer.

The effect of tacking down the bisubstrate analog at two points by virtue of the two Leu-124 main-chain hydrogen bonds is to place the sulfur atom within hydrogen bonding distance of Tyr-168; this is also accomplished by the movement of Phe-56, which enforces the "S"-shaped conformation of bound CoA-S-acetyltryptamine. Mutation of Tyr-168 to Phe significantly reduces V_{max} and increases the K_M of $AANAT_{28–201}$ (Table 2), indicating that this residue plays an important role in catalysis. This is the only mutation of an active site residue we have identified to date that has such a dramatic effect on enzymatic activity. Although the specific function of Tyr-168 is still unclear, the location of the −OH group within hydrogen bonding distance of the sulfur atom of CoA and the effect of its removal on catalysis suggest a role in the reprotonation of the thiolate leaving group. It is also possible that the −OH group could serve to correctly position the two substrates for catalysis. Catalytic tyrosine residues have been characterized in a number of enzymes (Rushmore and Pickett, 1993; Esposito and Scocca, 1997), and a group with a pKa of ~8.5 has been previously implicated in AANAT catalysis (Khalil et al., 1998). A schematic of the proposed reaction pathway is shown in Figure 5B.

Conclusions

The structure presented here allows us to compare two structural states for AANAT during catalysis. Our study reveals that, upon binding a bisubstrate analog, there is a large conformational change associated with one of the three surface loops, which is required to allow access of AcCoA to its binding site. Furthermore, this conformational change leads to the formation of the binding site for the second substrate in the reaction. We are not aware of another acetyltransferase for which such a structural rearrangement upon AcCoA binding has been characterized; in fact, in cases of other acetyltransferases where the structures of uncomplexed and complexed forms have been determined, such as chloramphenicol acetyltransferase (Leslie et al., 1988) or the hexapeptide xenobiotic acetyltransferase (Beaman et al., 1998), there is no significant protein movement upon AcCoA binding. It is possible that enzyme activity might be controlled through regulation of the capacity of AANAT to undergo such an AcCoA-induced conformational change.

Thus, it appears that the catalytic mechanism of AANAT involves cycling through several distinct structural states, starting from an unbound form in which a mobile part of the protein partially blocks the AcCoA-binding site. Upon binding, AcCoA displaces this region of loop 1, an event which is accompanied by a rearrangement of the residues in the loop. The result of this conformational change is to bring several hydrophobic residues into proximity of others contributed by the two other loops, and together, these form the serotonin-binding site. The binding of serotonin is likely stabilized by the resultant hydrophobic packing. We suggest that the reversal of this cycle after transfer of the acetyl group is driven by the increased hydrophobicity of N-acetylserotonin relative to serotonin in the vicinity of the acetylated amine group, which in turn helps the product leave its binding site. The empty serotonin-binding site is likely to lose its stability, and the mobile segment of the protein could revert to the conformation seen in the uncomplexed molecule, an event necessarily accompanied by ejection of CoA. It is not clear if the conformational change observed upon CoA-S-tryptamine binding by AANAT is a fundamental property of all the acetyltransferases in the GNAT superfamily, as this issue awaits further structural information on other members of the family.

Experimental Procedures

Expression and Protein Purification

The cloning, expression, and purification of truncated sheep $AANAT_{28–201}$ have been previously described (Hickman et al., 1999). For mutagenesis studies, point mutants were either prepared by Veritas, Inc. (Rockville, MD) or constructed using the QuikChange (Stratagene) method. To confirm that the desired mutation had been introduced and that no other codons had been changed, DNA sequencing was carried out on all plasmids using dye terminator cycle sequencing (ABI/Perkin Elmer) performed on a ABI 310 Capillary Genetic Analyzer. All fusion proteins used in this study were expressed on a 2 l scale and purified in the same manner as previously described for $AANAT_{28–201}$. Upon elution from glutathione-Sepharose columns, fractions containing each fusion protein (judged by absorption at 280 nm) were combined and dialyzed overnight against buffer 1 [20 mM Tris [pH 7.5], 0.5 M NaCl, 5 mM DTT, 1 mM EDTA,

10% (w/v) glycerol], which had been previously sparged with helium. This and subsequent steps were carried out at 4°C.

To remove coeluting chaperonin proteins from GST-NAT fusion proteins, gel filtration was performed on a Pharmacia SmartSystem Superdex 200 column equilibrated in buffer 1; this resulted in the complete separation of the fusion protein from the chaperone proteins. Fractions containing only the fusion protein (as judged by SDS–PAGE) were combined and dialyzed overnight against two changes of buffer 2 (50 mM citrate [pH 6.5], 0.3 M NaCl, 5 mM DTT, 1 mM EDTA, 10% [w/v] glycerol). The final protein concentration was determined by measuring the absorption at 280 nm and using the appropriate calculated extinction coefficient; purified fusion proteins were aliquoted into small volumes for subsequent use in enzyme assays and stored at −80°C. It had been previously demonstrated that the fusion protein has kinetic properties essentially identical to those of free AANAT (De Angelis et al., 1998).

Determination of Enzyme Activity

Enzyme activity was determined radiochemically by incubating 10 mM tryptamine and 0.5 mM ^{3}H-AcCoA (S.A. = 4 Ci/mol) for 15 min at 30°C and extracting the product, [^{3}H-acetyl]tryptamine, into chloroform (Namboodiri et al., 1987; Coon et al., 1995). To ensure that the mutant proteins were stable under assay conditions, time courses were performed (0 to 20 min), and linear product formation as a function of time was confirmed for the wild-type protein and all site-directed mutants. To determine K_M and V_{max} values, the enzyme assay was performed using varying concentrations of tryptamine. Each activity measurement was performed at least twice, and average values (which were within 10% of the mean) are reported in Table 2. Appropriate enzyme concentrations were chosen to ensure that less than 10% of the substrate was used up even at the highest substrate concentration. K_M and V_{max} values were calculated using nonlinear regression analysis (Kaleidagraph).

Confirmation of Coenzyme A-S-Acetyltryptamine Binding to $AANAT_{28-201}$

Coenzyme A-S-acetyltryptamine was provided by RBI (Natick, MA) as part of the Chemical Synthesis Program of the National Institute of Mental Health, contract NOIMH30003. It was purified to >97.9% by HPLC and characterized by ^{1}H NMR, ^{31}P NMR, and mass spectrometry. To determine conditions for binding to $AANAT_{28-201}$, the protein and CoA-S-acetyltryptamine were mixed in a 1:1 molar ratio in protein buffer, incubated at room temperature for 20 min, and the mixture applied to an analytical gel filtration Superdex 75 column run at 20°C. Only one peak was observed, with a A_{280}/A_{260} ratio corresponding to the expected sum of absorbances due to the protein and bisubstrate analog. Incubation of protein and CoA-S-acetyltryptamine in a 1:2 molar ratio resulted in two peaks eluting from the gel filtration column, one corresponding to the previously observed peak attributed to the complex, and one corresponding in position and intensity to the bisubstrate analog alone. Thus, under the buffer conditions used, $AANAT_{28-201}$ and CoA-S-acetyltryptamine form a 1:1 complex that is sufficiently stable to persist under gel filtration conditions.

Crystallization, Data Collection, and Structure Determination

$AANAT_{28-201}$ at 0.55 mM in buffer 2 was incubated at room temperature for 20 min with the bisubstrate analog at a final concentration of 1.0 mM. Clusters of thin plates grew overnight at 4°C in hanging drops consisting of a 1:1 mixture of the protein–analog complex and a well solution consisting of 30% (w/v) PEG 4000, 0.2 M ammonium sulfate (AS). Single crystals of sufficient size for data collection were obtained by three successive rounds of macroseeding. To confirm the presence of the bisubstrate analog, crystals were washed several times in water and then dissolved in 5% (v/v) acetic acid. Mass spectrometry measurements confirmed the mass of the protein (measured = 19,658 Da; calculated = 19,672 Da) and intact CoA-S-acetyltryptamine (measured = 966.5 Da in negative ion mode; calculated = 967.8 Da) with no obvious degradation products. For data collection, crystals were transferred to a stabilization solution consisting of 12.5% (w/v) PEG 4000, 0.1 M AS, 0.15 M NaCl, 25 mM citrate (pH 6.5), 0.5 mM EDTA, 5 mM DTT, 0.5 mM CoA-S-acetyltryptamine, and 5% (w/v) glycerol. The glycerol concentration was slowly increased to 22.5%, and the crystals were frozen by rapid immersion in liquid propane.

Data were collected at 95 K on a Raxis IV image plate detector mounted on a Rigaku RUH3R rotating anode source operated at 50 kV/100 mA with double mirror–focused Cu Kα radiation. The crystals belong to the space group $C222_1$ with cell dimensions a = 52.8 Å, b = 67.4 Å, c = 88.1 Å. There is one monomer in the asymmetric unit, and the crystals have a solvent content of 34.9%.

The crystal structure was solved with molecular replacement using AMoRe (Navaza, 1994). The search model was the uncomplexed form of the enzyme (Hickman et al., 1999; PDB ID code 1b6b). Because significant conformational changes were expected between residues 57 and 68, this segment was removed from the search. The diffraction data used were between 15 and 4 Å. The correlation coefficient of the rotation solution was 12.2% on amplitudes; after rigid body optimization, the best translation solution had a correlation coefficient of 34.1% and a crystallographic R factor of 48.6%. Further rigid body, molecular dynamics, energy minimization, and restrained B factor refinement were carried out with XPLOR 3.1 (Brünger, 1992a), gradually extending the resolution to 1.8 and 25 Å on the high and low ends, respectively, and completing the model. Five percent of the data, randomly selected, was used exclusively to monitor the free R factor (Brünger, 1992b). Bulk solvent correction was used and also the TNT B factor restraint library (Tronrud, 1996). Difference electron density for CoA-S-acetyltryptamine was clearly visible even at the early stages. The force field restraining the bisubstrate analog was composed of the parameter sets compiled by Engh and Huber (1991) and Parkinson et al. (1996) where appropriate. For the remaining parameters, the Cambridge Structural Database (CSD) was consulted for representative small-molecule crystal structures. At the end of the refinement, 223 water molecules were added.

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Coordinates have been deposited with the ID code 1cjw and are available until release via e-mail.