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Identification of catalytically distinct arylalkylamine *N*-acetyltransferase spliciforms from *Tribolium castaneum*

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Abstract

The assumption that structural or sequential homology between enzymes implies functional homology is a common misconception. Through in-depth structural and kinetic analysis, we are now beginning to understand the minute differences in primary structure that can alter the function of an enzyme completely. Alternative splicing is one method for which the activity of an enzyme can be controlled, simply by altering its length. Arylalkylamine *N*-acetyltransferase A (AANATA) in *D. melanogaster*, which catalyzes the *N*-acetylation of biogenic amines, has multiple spliciforms – alternatively spliced enzyme isoforms – with differing tissue distribution. As demonstrated here, AANAT1 from *Tribolium castaneum* is another such enzyme with multiple spliciforms. A screening assay was developed and utilized to determine that, despite only a 35 amino acid truncation, the shortened form of *Tc*AANAT1 is a more active form of the enzyme. This implies regulation of enzyme metabolic activity via alternative splicing.

Keywords

AANAT; transferase; acylation; *Tribolium*

Introduction

The Gcn5-related *N*-acetyltransferase (GNAT) superfamily of enzymes are known to catalyze the acyl-CoA-dependent acylation of an amine or arylalkylamine [1,2]; examples include the arylalkylamine *N*-acyltransferases (AANATs) [3,4], *N*-acetylglutamate

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Authors' Contributions

B.G.O., K.C.P., B.A.S., V.E.F., and G.S. performed the experiments detailed herein; B.G.O. and D.J.M. designed the experiments, monitored the progress of the research, and mentored the research team, wrote the manuscript, and obtained the funds for the work; and all the authors read and edited drafts of the manuscript, leading to the creation of the final article.

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Conflicts of interest

There are no conflicts to declare.

synthetases [5], and the histone acetyltransferases [6]. Genomic screens have shown that multiple GNAT-like enzymes could be expressed in an organism [7–9]. One possible reason for the multiplicity of GNAT enzymes in an organism lies in the array of functions mediated by these enzymes [6,10–16]. In mammals for example, only one AANAT has been identified, serotonin *N*-acetyltransferase (SNAT) [11,12], an important regulator of mammalian circadian rhythm. Insects on the other hand express an abundance of AANAT and AANAT-like enzymes. This is consistent with the understanding that insect AANATs (iAANATs) perform a variety of roles, including cuticle sclerotization, biogenic amine removal, and fatty-acid amide biosynthesis [14–18]. Much effort has been put into identifying and characterizing these enzymes and the panel of substrates each utilizes, from both an acyl-CoA donor and amine acceptor perspective [7,17,19–35].

Generally, iAANATs exhibit <40% amino acid sequence homology, but demonstrate remarkable structural conservation [36]. Four conserved sequence motifs and a common fold core are found for all GNAT enzymes, despite the low pairwise sequence identity [1]. Additionally, many of the iAANATs characterized to date have demonstrated significant variation in substrate specificity, especially regarding the amine acceptor. While this might have been expected between enzymes that share little sequence similarity, it is becoming clear that even variants of the same gene, expressed through alternative splicing, can serve different metabolic purposes.

Recently, an iAANAT from another insect, *Tribolium castaneum*, was shown to have a key role in cuticle sclerotization and pigmentation [25]. The enzyme, *TcAANAT1*, catalyzed the formation of acetylated amines with dopamine, tryptamine, norepinephrine, tyramine and octopamine functioning as the acceptor substrate. A BLAST search of the *T. castaneum* genome using *TcAANAT1* as a template identified just one additional discrete *TcAANAT*, the recently characterized *TcAANAT0* [37]. Also identified were several isoforms of *TcAANAT1*. One such isoform is a truncated version of the enzyme, 24 amino acids shorter, which we have named *TcAANAT1b*. We set about examining the effect of truncation on catalytic efficiency and substrate specificity. To do this, we utilized a convenient and straightforward strategy to identify substrates for GNAT enzymes and any other enzymes that utilize acyl-CoA thioesters as acyl donors. The screening strategy identified acyl-CoA and amine substrates accepted by *TcAANAT1b*. Upon the identification of substrates for *TcAANAT1b*, we found that truncation of *TcAANAT1* to yield *TcAANAT1b* results in a catalytically distinct version of the enzyme with significantly higher levels of activity. Through sequence and structural analysis, we then examined the reasoning behind this contrast.

Experimental

Cloning, Expression, and Purification of *TcAANAT1b*

The codon-optimized *TcAANAT1b* gene for *E. coli* expression including an N-terminal His₆ tag (accession no. NP_001139379.1) was synthesized commercially by Genscript and ligated into a *pET-28a* vector using *XhoI* and *NdeI* restriction enzymes using a New England Biolabs Quick Ligation™ kit. The vector was transformed into *E. coli* BL21 (DE3) competent cells for subsequent protein expression. The transformed cells were cultured at

37°C in 20g/L LB media supplemented with kanamycin (50 µg/mL) until an OD₆₀₀ of 0.6 was achieved. Protein expression was induced with the addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was left at 30°C for 5 hours. The cultures were then centrifuged at 5000 × g for 10 min at 4 °C and the resulting pellet was resuspended in 20 mM Tris, pH 8.0, 500 mM NaCl, and 5 mM imidazole. The cells were lysed via sonication and centrifuged at 10,000 × g for 20 min at 4°C. The supernatant containing the *N*-terminal His₆-tagged *TcAANAT1b* was collected and left rocking in 6 mL of HisPur nickel-nitrilotriacetic acid (Ni-NTA) chelating resin. The resin with the bound protein was packed into a column and equilibrated with 10 column volumes (CVs) of 20 mM Tris, pH 8.0, 500 mM NaCl, and 5 mM imidazole followed by a wash of 20 CVs of 20 mM Tris, pH 8.0, 500 mM NaCl, and 60 mM imidazole. The purified *TcAANAT1b* was eluted in 1.0 mL fractions with 20 mM Tris, pH 8.0, 500 mM NaCl, and 500 mM imidazole. The protein concentration in each fraction was measured using the Bradford assay and the protein dialyzed for 16 hours at 4°C against 20 mM Tris, pH 7.9, and 200 mM NaCl. Following dialysis, the samples were assayed again for concentration and the purity was assessed via SDS-PAGE gel.

Substrate Identification for *TcAANAT1b*

Once purified enzyme was available, the acyl-CoA and amine specificities of *TcAANAT1b* were established. We have a collection of 96 primary amines for evaluation as possible *TcAANAT1b* substrates, but to test each amine individually using even a single acyl-CoA would be cost-prohibitive and labor intensive. Instead, the amines were grouped together in screening pools of four based primarily on their molecular weight and chemical classification. For example, aminoglycosides were allocated to one pool, while catecholamines, were similarly grouped together. The final concentration of each amine in the pooled solutions was initially 100 mM.

The *N*-acyltransferase catalyzed acylation of an amine results in two products, the *N*-acylamide and coenzyme A (CoA-SH). Formation of CoA-SH was quantified spectrophotometrically using a reporter molecule, 4,4'-dithiodipyridine (DTDP). The *in vitro* formation of CoA-SH results in reduction of the disulfide bond of DTDP to the corresponding thioketone ($\epsilon = 19,800 \text{ M}^{-1}\text{cm}^{-1}$ at pH 8.0 [38]), the rate of which can be measured at 324 nm.

To perform the screening assay, each pool was tested individually at 30°C in presence of a saturating concentration of either acetyl-CoA (short acyl chain: 2 carbons) or oleoyl CoA (long acyl chain: 18 carbons). A reaction volume of 750 µL consisted of 300 mM Tris (pH 8.0), 375 µM DTDP, 250 µM acetyl or oleoyl CoA, and 20 mM of the screening pool. Reactions were initiated by the addition of 320 nM enzyme and the rate of change of absorbance per second was recorded.

A simple method to determine a hit is to measure the background rate of hydrolysis of the acyl-CoA and to set a standard of 10× that rate. To validate this assay further, we measured the Z-factor (Equation 1 - σ_S and σ_C denote the sample and background signal standard deviation, and $\bar{x}_S$ and $\bar{x}_C$ denote the sample and mean background signal, respectively) [39]. This measurement is used to determine the viability of an assay for high-throughput

screening. However, we found that it equally effective in determining a hit from a pool of substrates. A score between 0.5 and 1 indicates a pool that merits individual examination. Any value below 0.5 would not be a hit.

$$Z = 1 - \frac{(3\sigma_S + 3\sigma_C)}{|\bar{x}_S - \bar{x}_C|} \quad \text{Equation 1}$$

A successful screen was followed up by the testing of the individual amines of the pool, independently. This screening procedure was repeated for all the pools of amines until a panel of amine substrates was identified. From here, individual kinetic parameters were measured using the same spectrophotometric technique, while varying substrate concentration. Also, this method was used in a similar fashion to test different acyl-CoAs. The concentration of histamine was held constant, while varying the concentration of a range of acyl-CoAs to determine if any long-chain acyl-CoAs, branched-CoAs, carboxylic-CoAs, or benzylated-CoAs act as donor substrates.

Results and Discussion

Production of Recombinant *TcAANAT1b* in *E. coli*.

TcAANAT1b, codon-optimized for *E. coli* expression was ligated into a *pET-28a* vector and the resulting *TcAANAT1b pET-28a* vector was transformed into *E. coli* BL21 (DE3) cells. The N-terminus His₆-tagged *TcAANAT1b* was purified using Ni-NTA affinity resin yielding 7.0 mg/L of culture of homogeneous enzyme. Purity of *TcAANAT1b* was assessed by SDS-PAGE, with a single band of the proper molecular weight (28 kDa) indicating 95% purity (Figure 1).

Characterization of Amine Substrates for Recombinant *TcAANAT1b*

Due to the large panel of amine substrates examined, a selection of three pools were tested with acetyl-CoA as the co-substrate (Table 1).

As defined by Equation 1, Pool 1 and 2 demonstrated activity above the control threshold. Next, each amine of Pool 1 and 2 was individually examined to determine which served as substrates for the enzyme.

In total, eight amines were found to have activity with *TcAANAT1b* with acetyl-CoA acting as donor: dopamine, octopamine, serotonin, 2-phenethylamine, norepinephrine, 2,2-difluoro-2-phenethylamine, histamine, tryptamine and tyramine. This panel of amine substrates suggests that *TcAANAT1b* has a role in the inactivation of biogenic amine neurotransmitters. Their respective kinetic constants are presented in Table 2. While tryptamine and tyramine were substrates, significant substrate inhibition was observed at concentrations approaching the threshold of sensitivity for the reporter molecule, making it difficult to accurately measure the kinetic constants for these two amines.

Dopamine exhibited the highest k_{cat}/K_M ratio for the amine substrates we identified for *TcAANAT1b*, consistent with work demonstrating that *TcAANAT1* potentially has a role in cuticle morphology and pigmentation [25].

We found that both enzymes share five amines as substrates: dopamine, octopamine, tryptamine, tyramine and norepinephrine, the respective kinetic constants are different for these enzymes. In addition to the five shared amine substrates, we identified four other amines that serve as *TcAANAT1b* substrates: 2-phenethylamine, 2,2-difluoro-2-phenethylamine, and histamine. It is unknown if these amines are substrates for *TcAANAT1*.

A direct comparison between the kinetic constants shared between the enzymes is feasible because those reported for *TcAANAT1* (Table 3) were measured under conditions similar to those used to measure the values for *TcAANAT1b* (Table 2). Differences between K_M values for the two spliciforms are relatively small; all are within a factor of 10 for these two *TcAANAT1* variants. However, the k_{cat} values for *TcAANAT1* are 100- to 400-fold lower relative to those measured for *TcAANAT1b*.

Characterization of Acyl-CoA Substrates for Recombinant *TcAANAT1b*

To examine the capacity of *TcAANAT1b* to catalyze amine acylation, a panel of acyl-CoAs were tested for activity while the concentration of histamine was held constant. Histamine was selected because many of the other substrates (namely the catecholamines) were found to have an inhibitory effect at relatively low concentrations. We tested acetyl-CoA, butyryl-CoA, decanoyl-CoA, palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, arachidonyl-CoA, malonyl-CoA, and benzoyl-CoA as *TcAANAT1b* substrates.

Of this set of acyl-CoAs, only acetyl-, butyryl-, and decanoyl-CoA were substrates (Table 4). Comparison of the kinetic data for the acyl-CoAs between the two *TcAANAT1* variants is not feasible because similar data for *TcAANAT1* is not available. The decrease in the K_M values from acetyl-CoA to decanoyl-CoA is approximately matched with a decrease in the k_{cat} values; thus, we find little change in the k_{cat}/K_M values. These data are consistent with our earlier work on other iAANATs showing that long-chain acyl-CoAs are inhibitors, competitive vs. acetyl-CoA [19,22,24]. These data suggest that the amine binding pocket is blocked if the acyl chain is beyond a certain length.

Importance of the AANAT N-terminus

It is apparent that the deletion of 35 amino acids from the N-terminus of *TcAANAT1* to produce *TcAANAT1b* yields an enzyme of higher activity. The k_{cat} values for *TcAANAT1* are 100-fold higher than the values for *TcAANAT1b* (compare Tables 2 and 3). Deletion of the 35 amino acids in the conversion of *TcAANAT1* → *TcAANAT1b* has little effect on amine substrate binding, within the limits of how accurately K_M represents K_d (the true dissociation constant). Thus, the reasons for the significant increase in k_{cat} upon the truncation of *TcAANAT1* to *TcAANAT1b* could be an increased affinity for the acyl-CoA, an increase in the rate of product formation, or combination of all these possibilities.

Studies of the kinetic mechanism for most AANATs show that the first substrate to bind is the acyl-CoA. Acyl-CoA binding induces a conformational change that converts the amine binding pocket from a low affinity to a high affinity state. AANAT activity, as measured by the k_{cat} , is highest in the high affinity conformation.

As shown in Figure 2 (underlined), the catalytic site of the amine binding pocket is often located relatively close to the *N*-terminus in AANATs. While there is no structure yet available for *Tc*AANAT1, several other iAANAT structures have been reported: *T. castaneum* (*Tc*AANAT0) [37], *D. melanogaster* (*Dm*AANAT0) [40], and *A. aegypti* (*Aa*NAT2, *Aa*NAT5b and *Aa*NAT7) [41], as well as mammalian SNAT [42,43]. SNAT has the longest *N*-terminus before amine binding pocket, approximately 60 residues long. The structure of *N*-terminal region in SNAT is a β -sheet wrapped by two α -helices of different lengths [43]. This structural motif begins at residue 70 in *Tc*AANAT1.

Despite the existence of several iAANAT structures with ligands bound, not one apo-structure of an iAANAT has been reported. To examine the conformational changes associated with acetyl-CoA binding, the apo and holo structures for SNAT were considered. Upon acetyl-CoA binding, much of the SNAT structure (~95%) retains its basic shape except for the *N*-terminus, which undergoes a significant change. A motif stretching from 51 to 67 is a disordered region in apo-SNAT and this region stretches into the yet unformed amine binding pocket. Upon acetyl-CoA binding, this region organizes into the amine pocket α -helix, as shown in the structural alignment of free SNAT (PDB: 1B6B [43]) with that bound to the bisubstrate analog CoA-S-acetyl-5-bromotryptamine (PDB: 1KUV [42]) (Figure 3). The structural similarities between AANATs implies that a similar mechanism could occur in *Tc*AANAT1 and other iAANATs. The extension of the *N*-terminus in *Tc*AANAT1 may have a detrimental effect on this conformational shift. A crystal structure of the bound and unbound forms of *Tc*AANAT1 must be obtained to more fully address this point.

Conclusion

Through the use of a well-validated and cost-effective screening assay, we have demonstrated the ability to quickly identify and characterize differences in kinetic ability, not just between different *N*-acetyltransferases, but between seemingly identical variants of the same *N*-acetyltransferases. This process allows us to quickly answer the basic questions of catalytic efficiency and begin the process of understanding the intrinsic differences in structure and chemical mechanism, which are the root origin for these differences. Using these methods, we have shown that the truncated variant of *Tc*AANAT1 is a more catalytically active form of the enzyme. As is often the case for alternative splicing, this is potentially a method to control the rate of acetylation of biogenic amines. In this sense, excess substrate or a scarcity of product results in an upregulation of the truncated form of the enzyme. These findings give further credence to the heterogeneity of iAANATs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Identification of truncated splicing form of arylalkylamine *N*-acetyltransferase 1 from *Tribolium castaneum*, named *TcAANAT1b*.
- Purification and characterization of *TcAANAT1b*
- Regulation of *TcAANAT1* activity by alternative splicing

Figure 1: Cloning and purification of TcAANAT1b.

(A) TcAANAT1b gene from Genscript (NCBI accession number: NM_001145907.1). Lane 1, 1 kb ladder; Lane 2, TcAANAT1b linear DNA pre-ligation (719 bp). (B) SDS-PAGE of purified TcAANAT1b protein. Lane 1, Thermo Scientific PageRuler Prestained Protein Ladder; Lane 2, purified TcAANAT1b (27.8 kDa).

Figure 2:
Sequence alignment of AANAT1 (NCBI reference: XP_972873.2), AANAT1b (NP_001139379.1) and AANAT0 (XP_972873.2) from *Tribolium castaneum*, as well as AANATA (NP_523839.2) from *Drosophila melanogaster*

Figure 3:
Structure of SNAT with bisubstrate analog bound (pink/red) and without (green/blue). The blue and red motifs represent residues 51–67 which undergo significant organization upon initial substrate binding.

Table 1:
Screening results for three distinct pools of amines.

All units are in A/min

Pool	Pool contents	$\bar{x}_s$	σ_s	$\bar{x}_c$	σ_c	Z-Factor	Pass or fail?
1	Lysine Threonine Glycine Histamine	5.4	0.27	0.014	0.001	0.85	Pass
2	Serotonin Dopamine Tryptamine Norepinephrine	0.57	0.08	0.014	0.001	0.56	Pass
3	Aspartate Isoleucine Tryptophan Glutamine	0.043	0.004	0.014	0.001	0.42	Fail

Table 2:
Steady-state kinetic constants of most active amine substrates for TcAANAT1b.

Kinetic constants were measured using 300 mM Tris-HCl, pH 8.0, 375 μ M DTDP, and 250 μ M acetyl-CoA

Substrate	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Dopamine	9.7 ± 2.0	26 ± 1.2	$(2.7 \pm 0.5) \times 10^6$
Octopamine	18 ± 5.9	40 ± 5.1	$(2.2 \pm 0.7) \times 10^6$
Serotonin	42 ± 10	70 ± 11	$(1.7 \pm 0.1) \times 10^6$
2-Phenethylamine	81 ± 22	87 ± 14	$(1.1 \pm 1.1) \times 10^6$
Norepinephrine	53 ± 9.9	41 ± 2.5	$(7.7 \pm 1.5) \times 10^5$
2,2-Difluoro-2-phenethylamine	327 ± 43	85 ± 3.8	$(2.6 \pm 0.4) \times 10^5$
Histamine	$1,400 \pm 190$	59 ± 2.8	$(4.3 \pm 0.6) \times 10^4$

Table 3:

Kinetic constants for TcAANAT1. Kinetic constants are from Noh et al. [25].

<i>TcAANAT1</i>	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1}\text{s}^{-1}$)
Dopamine	110 ± 40	0.5 ± 0.1	$(4.5 \pm 0.2) \times 10^3$
Octopamine	70 ± 10	0.4 ± 0.06	$(5.7 \pm 1.2) \times 10^3$
Norepinephrine	50 ± 30	0.1 ± 0.03	$(2.0 \pm 1.3) \times 10^3$

Table 4:
Steady-state kinetic constants of acyl-CoA substrates for TcAANAT1b.

Kinetic constants were measured using 300 mM Tris-HCl, pH 8.0, 375 μ M DTDP, and 5 mM histamine

Substrate	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Acetyl-CoA	65 ± 9.8	52 ± 2.5	$(8.0 \pm 0.1) \times 10^5$
Butyryl-CoA	45 ± 4.9	14 ± 0.5	$(3.1 \pm 0.4) \times 10^5$
Decanoyl-CoA	6.7 ± 0.8	3.4 ± 0.1	$(5.1 \pm 0.6) \times 10^5$