Published in final edited form as:

Insect Biochem Mol Biol. 2015 November ; 66: 1–12. doi:10.1016/j.ibmb.2015.10.003.

Probing the Chemical Mechanism and Critical Regulatory Amino Acid Residues of *Drosophila melanogaster* Arylalkylamine *N*-acyltransferase Like 2

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Abstract

Arylalkylamine *N*-acyltransferase like 2 (AANATL2) catalyzes the formation of *N*-acylarylalkylamides from the corresponding acyl-CoA and arylalkylamine. The *N*-acylation of biogenic amines in *Drosophila melanogaster* is a critical step for the inactivation of neurotransmitters, cuticle sclerotization, and melatonin biosynthesis. In addition, *D. melanogaster* has been used as a model system to evaluate the biosynthesis of fatty acid amides: a family of potent cell signaling lipids. We have previously showed that AANATL2 catalyzes the formation of *N*-acylarylalkylamides, including long-chain *N*-acylserotonins and *N*-acyldopamines. Herein, we define the kinetic mechanism for AANATL2 as an ordered sequential mechanism with acetyl-CoA binding first followed by tyramine to generate the ternary complex prior to catalysis. Bell shaped $k_{cat,app}$ – acetyl-CoA and $(k_{cat}/K_m)_{app}$ – acetyl-CoA pH-rate profiles identified two apparent $pK_{a,app}$ values of ~7.4 and ~8.9 that are critical to catalysis, suggesting the AANATL2-catalyzed formation of *N*-acetyltyramine occurs through an acid/base chemical mechanism. Site-directed mutagenesis of a conserved glutamate that corresponds to the catalytic base for other *D. melanogaster* AANATL enzymes did not produce a substantial depression in the $k_{cat,app}$ value nor did it abolish the $pK_{a,app}$ value attributed to the general base in catalysis (pK_a ~7.4). These data suggest that AANATL2 catalyzes the formation of *N*-acylarylalkylamides using either different catalytic residues or a different chemical mechanism relative to other *D. melanogaster* AANATL enzymes. In addition, we constructed other site-directed mutants of AANATL2 to help define the role of targeted amino acids in substrate binding and/or enzyme catalysis.

Graphical abstract

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Keywords

AANATL2; chemical mechanism; structure-function relationships; *N*-acyldopamines; *N*-acylserotonins

1. Introduction

The biogenic arylalkylamines identified in *Drosophila melanogaster* consist of serotonin, dopamine, octopamine, and tyramine (Blenau and Baumann, 2011). Outlined in Scheme 1 is the metabolic flow of tyrosine to *N*-acyltryramine, *N*-acyloctopamine, and *N*-acyldopamine (Pathway A) and tryptophan to *N*-acylserotonin (Pathway B). Each biogenic amine and their respective *N*-acylated product are derived from the cognate amino acid. Two general reactions found in each pathway shown in Scheme 1 are amino acid decarboxylation and *N*-acylation. L-Amino acid decarboxylase is the decarboxylase involved in these pathways and is a relatively well understood (Han et al., 2010). The arylalkylamine *N*-acyltransferases (AANATs) catalyzing the acyl-CoA-dependent *N*-acylation reactions shown in Scheme 1 are likely members of the GCN5-related *N*-acetyltransferase (GNAT) superfamily of enzymes (Dyda et al., 2000; Vetting et al., 2005). However, the role played by the AANATs *in vivo* is not fully appreciated. Much of the focus on the AANATs has centered on their function in amine *N*-acetylation because the *N*-acetyl derivatives of most biogenic amines are known metabolites and *N*-acetylation is important in neurotransmitter inactivation, cuticle sclerotization, and melatonin biosynthesis (Andersen, 2010; Brunet, 1980; Finocchiaro et al., 1988; Wright, 1987).

AANATs represent a fascinating family of enzymes from an evolutionary perspective. The AANAT family, all belonging to the GNAT superfamily of acyltransferases, is comprised of the insect AANATs, (iAANATs), the non-vertebrate AANATs (NV-AANATs), and the vertebrate AANATs (VTAANATs) (Coon and Klein, 2006; Falcón et al., 2014; Hiragaki et al., 2015; Pavlicek et al., 2010). The division of the AANAT family of enzymes into iAANATs, NV-AANATs, and VT-AANATs is based on sequence and phylogenetic analyses. The VT-AANATs function primarily in the melatonin metabolism and changes in VT-AANAT activity correlate to the rhythmic production of melatonin in the pineal gland (Klein, 2006). Hence, VT-AANAT has been called the “timezyme” and the evolution of the timezyme seems intimately tied to pineal gland and vertebrate photodetection (Falcón et al., 2014; Klein, 2007). The NV-AANATs function broadly in arylalkylamine detoxification and also in DNA biology resulting from polyamine acetylation (Ganguly et al., 2001; Pavlicek et al., 2010; Wittich and Walter, 1990). In fact, arylalkylamine acetylation is thought to have played a role in primitive photodetection by protecting retinaldehyde, leading to the proposal that VT-AANAT evolved from NV-AANAT as the pineal gland evolved from a primitive photosensor (Falcón et al., 2014). By virtue of their ability to

acetylate melatonin, dopamine, and other biogenic amines, iAANATs are important in sclerotization, photoperiodism, amine detoxification, and cell signaling (Dempsey et al., 2014b; Hiragaki et al, 2015). The expression of an array of iaaNATs in a specific insect, as many as seven in *Drosophila* (Amherd et al, 2000; Dempsey et al., 2014a) and 13 in *Aedes* (Mehere et al., 2011) as examples, further complicates our understanding of the iaaNATs. The issue of multiple AANATs is important in organisms other than insects as amphioxus express seven NB-AANATs (Pavlicek et al., 2010) and teleost fish express three VT-AANATs (Cazaméa-Catalan et al., 2014; Coon and Klein, 2006). One important step in better understanding the various AANATs is a careful and thorough characterization of these enzymes individually, particularly in consideration of their potential involvement in fatty acid amide biosynthesis.

We have long thought that an AANAT or AANAT-like (AANATL) enzyme would be involved in biosynthesis of long-chain *N*-fatty acid amides (Merkler et al., 1995), a diverse family of cell signaling lipids (Farrell and Merkler, 2008; Fezza, et al., 2014). The identification of glycine *N*-acyltransferase-like enzymes that catalyze the formation of *N*-fatty acylglycines (Waluk et al., 2010) and our discovery that AANATL2 will catalyze the formation of *N*-fatty acylserotonins and *N*-fatty acyldopamines support our hypothesis. *D. melanogaster* is an excellent model organism for the study of the fatty acid amides, the AANAT-like enzymes, and their putative role in fatty acid amide metabolism. This organism is known to produce fatty acid amides (Jeffries et al., 2014; Tortoriello et al., 2013); therefore, one of our goals is to clone, over-express, and characterize all of these putative *D. melanogaster* *N*-acyltransferases and, ultimately, define how these enzymes contribute to fatty acid amide metabolism the flies. Our earlier work strongly suggests that AANATL2 is involved in the generation of *N*-fatty acylserotonins and *N*-fatty acyldopamines in *D. melanogaster* *in vivo* (Dempsey et al., 2014b). In addition, AANATL2 will accept a broad set of short-chain acyl-CoA thioesters as substrates, providing convenient tools for the study of its kinetic and chemical mechanisms.

Herein, we report the characterization of *D. melanogaster* AANATL2 that has been over-expressed in *E. coli*. We define the amine substrate specificity of the enzyme, have solved the kinetic mechanism by using double reciprocal analysis and dead-end inhibition, and propose a chemical mechanism for catalysis based on pH-rate studies and the results of site-directed mutagenesis experiments of targeted amino acid residues.

2. Material and methods

2.1 Materials

Antarctic Phosphatase, T4 DNA ligase, *Nde*I, and *Xho*I were purchased from New England Biolabs; XL10 *E. coli* cells, the *pET-28a(+)* vector, and BL21 (DE3) *E. coli* cells were purchased from Novagen; kanamycin monosulfate and IPTG were purchased from Gold Biotechnology; acetyl-CoA and oleoyl-CoA were purchased from Sigma-Aldrich; PCR primers were purchased from Eurofins MWG Operon; PfuUltra High-Fidelity DNA polymerase was purchased from Agilent; and ProBond™ nickel-chelating resin was purchased from Invitrogen. Wild-type AANATL2 was expressed and purified using the

same procedures as published by Dempsey *et al.* (Dempsey et al., 2014b). All other reagents were of the highest quality available from either Sigma-Aldrich or Fisher Scientific.

2.2 Cloning, expression, and purification of AANATL2 site-directed mutants

All the AANATL2 mutants were generated by using the overlap extension method (Ho et al., 1989). PCR primers (Table S1) were generated for each mutant by using the Agilent QuickChange Primer Design tool. The following PCR conditions were used with PfuUltra High-Fidelity DNA polymerase: initial denaturing step of 95°C for 2 min, then 30 cycles of (95°C for 30 s; 60°C for 30 s; 72°C for 1 min), and a final extension step of 72°C for 10 min. The AANATL2 mutant PCR product was then inserted into a *pET-28a(+)* vector using *NdeI* and *XhoI* restriction sites. The AANATL2-mutant-*pET-28a* product was transformed into *E. coli* XL10 competent cells, plated on Luria Broth (LB) agar plates supplemented with 40 µg/mL kanamycin, and incubated overnight at 37°C. The following day, a single colony from the LB agar plate containing the *E. coli* XL10 cells with the AANATL2-mutant-*pET-28a* vector was used to inoculate an overnight culture in LB media, supplemented with 40 µg/mL kanamycin at 37°C. The next day, the AANATL2-mutant-*pET-28a* plasmid was purified using Promega Wizard® Plus SV Minipreps DNA purification kit and sequenced by Eurofins MWG operon. Following confirmation of the AANATL2 mutant sequence, the AANATL2-mutant-*pET-28a* plasmid was then transformed into BL21 (DE3) *E. coli* cells. Each AANATL2 mutant protein was expressed and purified using the same procedures that were defined for the wild-type enzyme (Dempsey et al.).¹¹ After the purification of each enzyme, the AANATL2 mutants and wild-type enzyme were dialyzed into 20 mM Tris pH 7.4, 200 mM NaCl for 16 hr at 4°C.

2.3 Enzyme assay

Ellman's reagent (DTNB) was used to determine the kinetic constants for different amino donor substrates and acetyl-CoA with AANATL2 by measuring the rate of coenzyme A release at 412 nm (molar extinction coefficient = 13,600 M⁻¹ cm⁻¹) (Ellman, 1959) at 22°C and measuring the initial velocities with a Cary 300 Bio UV-Visible spectrophotometer. The assay solutions consisted of 300 mM Tris pH 8.0, 150 µM DTNB, varying concentration of one substrate while holding the other substrate at a fixed saturating concentration. The resulting data was fit to equation 1, in which v_o is the initial velocity, V_{max} is the maximal velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis constant. Each assay was performed in triplicate and the uncertainty for the $k_{cat,app}$ and $(k_{cat}/K_m)_{app}$ values were calculated by using equation 2 (Morrison and Uhr, 1966), for which σ is the standard error.

$$v_o = \frac{V_{max} [S]}{K_m + [S]} \quad \text{Equation 1}$$

$$\sigma \left(\frac{x}{y} \right) = \frac{x}{y} \sqrt{\left(\frac{\sigma_x}{x} \right)^2 + \left(\frac{\sigma_y}{y} \right)^2} \quad \text{Equation 2}$$

2.4 Kinetic mechanism

Initial velocity patterns for acetyl-CoA and tyramine were used to distinguish between the sequential or ping-pong kinetic mechanisms. The initial velocities were measured for acetyl-CoA by holding the tyramine concentration constant (1 μ M, 2 μ M, 10 μ M, and 25 μ M) and varying the concentration of acetyl-CoA. The initial velocities for tyramine were measured by holding the acetyl-CoA concentration constant (1 μ M, 2 μ M, 10 μ M, and 25 μ M) and varying the concentration of tyramine. The resulting data were fit to equation 3 for a Bi Bi sequential kinetic mechanism and plotted as a double reciprocal plot using IGOR Pro 6.34A. In equation 3, K_{ia} is the dissociation constant for substrate A (acetyl-CoA), K_a is the K_m (Michaelis constant) for substrate A (acetyl-CoA), and K_b is the K_m (Michaelis constant) for substrate B (tyramine).

$$v = \frac{V_{max} [A] [B]}{K_{ia}K_b + K_a [B] + K_b [A] + [A] [B]} \quad \text{Equation 3}$$

Following the double reciprocal analysis, dead-end inhibitor analysis was used to differentiate between an ordered or random sequential kinetic mechanism. Oleoyl-CoA and tyrosol were used as dead-end inhibitors as these are analogs for acetyl-CoA and tyramine, respectively. Initial velocities for the inhibition experiments were measured for acetyl-CoA and tyramine by varying the concentration of one substrate, holding the other substrate at a fixed concentration equal to the respective $K_{m,app}$ (acetyl-CoA = 7.2 μ M; tyramine = 3.9 μ M), at different concentrations of the inhibitor. Inhibition patterns were determined by fitting the resulting data, using SigmaPlot 12.0, to equations 4 – 6; for competitive, noncompetitive, and uncompetitive inhibition, respectively. The terms in equations 4 – 6 represent the following: v_o is the initial velocity, V_{max} is the maximal velocity, $[S]$ is the substrate concentration, K_m is the Michaelis constant, $[I]$ is the inhibitor concentration, and K_i is the inhibition constant. Each assay was performed in triplicate.

$$v_o = \frac{V_{max} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad \text{Equation 4}$$

$$v_o = \frac{V_{max} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad \text{Equation 5}$$

$$v_o = \frac{V_{max} [S]}{K_m + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad \text{Equation 6}$$

2.5 Rate versus pH dependence

The pH dependence on the kinetic constants for the wild-type and E29A mutant enzymes were determined for acetyl-CoA. The kinetic constants were determined at pH values ranging from 6.5 – 9.5 at intervals of every 0.5 pH units. The following buffers were used for the pH dependence study: MES (pH 6.5 – 7.0), Tris (pH 7.0 – 9.0), and AMeP (pH 9.0 –

9.5). The kinetic constants from the pH dependence experiments were fit using IGOR Pro 6.34A, to equation 7 [$\log (k_{\text{cat}}/K_{\text{m}} - \text{tyramine})$] and equation 8 [$\log (k_{\text{cat}})$], where c is the pH-independent plateau. Each assay was performed in duplicate.

$$\log \left(\frac{k_{\text{cat}}}{K_{\text{m}}} \right) = \log \left[c / \left(1 + 10^{pK_{\text{b}} - \text{pH}} + 10^{\text{pH} - pK_{\text{a}}} \right) \right] \quad \text{Equation 7}$$

$$\log (k_{\text{cat}}) = \log \left[c / \left(1 + 10^{pK_{\text{b}} - \text{pH}} + 10^{\text{pH} - pK_{\text{a}}} \right) \right] \quad \text{Equation 8}$$

3. Results and discussion

3.1 Structure-activity relationships: amine substrates AANATL2

We have previously published a limited set of data regarding the substrate specificity of AANATL2 because our goal, in that study, was to correlate the identification of long-chain *N*-acylserotonins in *D. melanogaster* to substrates for the enzyme. We found that long-chain acyl-CoAs would only serve as AANATL2 substrates when the amine substrate was serotonin or dopamine. However, all the known biogenic arylalkylamines in *D. melanogaster* (serotonin, dopamine, octopamine, and tyramine) are AANATL2 substrates when acetyl-CoA is the acyl-CoA donor (Dempsey et al., 2014b). In the work we report here, we further evaluate the structure-activity relationships for other amino donor substrates with acetyl-CoA serving as the corresponding acyl-CoA substrate (Table 1). We focused on five major structural features for amino donor substrates consisting of: (a) modification of the of the indole ring for tryptamines, (b) modification of the phenyl ring for phenethylamines, (c) modification of the two methylene spacer between the phenyl ring and the primary amine in phenethylamine analogs, (d) alternations in the space length between the phenyl ring and the primary amine in phenethylamine analogs, and (e) other non-arylalkylamine amino donors.

Tryptamine (2-(1*H*-Indol-3-yl)ethanamine), the decarboxylated analog of tryptophan, possesses an indole ring. With the exception of 5-methoxytryptamine, the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ values the set of tryptamine analogs we evaluated as AANATL2 substrates were the same within experimental error (Table 1). The relatively small decrease in the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ value for 5-methoxytryptamine, 1.8 – 3.5-fold, relative to tryptamine and the other 5-substituted tryptamines resulted from an increase in the $K_{\text{m,app}}$ value. The $k_{\text{cat,app}}$ value was generally the same for all the tryptamine analogs we included, except for a ~2-fold higher value for tryptamine. In all, there were minimal differences between the kinetic constants for tryptamine and the 5-substituted tryptamine substrates, indicating that AANATL2 can readily accommodate even a large benzyloxy group at the 5-position of the indole ring. Accommodation could occur via a region in the AANATL2 active site or flexibility in the active site that does not significantly affect the effective positioning of the amine for nucleophilic attack at the carbonyl of the acetyl-CoA thioester moiety.

Phenethylamine, $\text{C}_6\text{H}_5\text{-CH}_2\text{-CH}_2\text{-NH}_2$, is a AANATL2 substrate exhibiting a similar $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ to that we measured for tryptamine. Included in our assessment of amine

substrates were six ring-substituted phenethylamines: 3-(trifluoromethyl)phenethylamine, 4-methoxyphenethylamine, 3-methoxyphenethylamine, 4-hydroxyphenethylamine (tyramine), 3,4-dihydroxyphenethylamine (dopamine), and 3,4-methylenedioxypheethylamine (Table 1). We found limited variation in the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ values between the six phenethylamines included in our study; the largest difference being the ratio of $(k_{\text{cat}}/K_{\text{m}})_{\text{app, 3-(trifluoromethyl)phenethylamine}}/(k_{\text{cat}}/K_{\text{m}})_{\text{app, dopamine}} \sim 3$.

Another class of phenethylamine analogs we evaluated as AANATL2 substrates, were variants in the $-\text{CH}_2-\text{CH}_2-$ spacer between the phenyl ring and the amine. Included in this group of phenethylamines are octopamine and norepinephrine with a β -hydroxyl group attached to the spacer, β -methylphenethylamine with a β -methyl group attached to the spacer, 4-phenylbutylamine with a longer spacer ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-$), and benzylamine with a shorter spacer ($-\text{CH}_2-$). For two of the phenylethylamine pairs that only differ in a β -substituent on the spacer, dopamine/norepinephrine and phenethylamine/ β -methylphenethylamine, we found little difference in the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ values. However, we did measure a ~ 10 -fold difference in $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ values for the tyramine/octopamine pair – a result that is difficult to reconcile with our other data. The decrease in the spacer length has a dramatic effect on catalytic efficiency as benzylamine is a very poor substrate when compared to phenethylamine, the ratio of $(k_{\text{cat}}/K_{\text{m}})_{\text{app, benzylamine}}$ to $(k_{\text{cat}}/K_{\text{m}})_{\text{app, phenethylamine}}$ is ~ 0.002 . In contrast, the increase in the spacer length from two methylene groups to four has a limited effect on catalytic efficiency, evidence being the ratio of $(k_{\text{cat}}/K_{\text{m}})_{\text{app, phenylbutylamine}}$ to $(k_{\text{cat}}/K_{\text{m}})_{\text{app, phenethylamine}}$ of is ~ 0.50 . In considering our data on the phenethylamines as AANATL2 substrates, we found that decoration of the phenyl ring or $-\text{CH}_2-\text{CH}_2-$ spacer and an small increase in the spacer length has limited effect on the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ values. These results, again, point toward flexibility in the AANATL2 active site to accommodate structural differences between the phenethylamine substrates, included in our study.

An obvious set of amine substrates to consider in our evaluation of potential amine substrates are the amino acids, possessing a carboxylate moiety at the α -position of the spacer. Tyrosine, phenylalanine, and tryptophan, the amino acids most related to arylalkylamine substrates reported in Table 1, were not AANATL2 substrates at a concentration of 1.0 mM, suggesting that the modification of the α -position of the spacer group precludes catalysis by either a steric effect, an electrostatic effect, or a combination of the two effects. To evaluate between these possibilities, we evaluated tyrosine methyl ester as a substrate and, again, found no activity at 1.0 mM, suggesting that the arylalkylamines with a modification at the α -position of the spacer group will not serve as substrates prominently due to a steric effect. We further found no inhibition of the AANATL2-catalyzed formation of *N*-acetyltyramine from acetyl-CoA and tyramine for tyrosine, phenylalanine, tryptophan, and tyrosine methyl ester at 1.0 mM, confirming that these α -position modified arylalkylamines do not bind or bind weakly to AANATL2 ($K_{\text{d}} \sim 10$ mM).

In completing our studies of potential amine substrates for AANATL2, we examined two non-arylalkylamines as substrates: histamine and ethanolamine. Histamine is substrate with a relatively high $k_{\text{cat}}/K_{\text{m}}$ for AANATL7, a related arylalkylamine *N*-acyltransferase in *D. melanogaster* (Dempsey et al., 2015) and the acylation of ethanolamine would lead to the

bioactive *N*-acylethanolamines (Farrell and Merkler, 2008). We found that AANATL2 will catalyze the *N*-acetylation of histamine and ethanolamine, albeit with high $(K_m)_{app}$ and low $k_{cat,app}$ values relative to the arylalkylamine substrates. For example, when compared to the arylalkylamine with the highest $(k_{cat}/K_m)_{app}$ value, the $(k_{cat}/K_m)_{app}$ values for histamine and ethanolamine are only 0.06% and 0.002% of that for 3-(trifluoromethyl)phenethylamine, respectively. We also determined that AANATL2 does not catalyze the *N*-acetylation of isoniazid, which is a first line drug for the treatment of tuberculosis (Chang et al., 2013; Vernon, 2013; Zumla et al., 2013). In sum, our data suggest that AANATL2 predominantly catalyzes the *N*-acetylation of biologically relevant arylalkylamines.

3.2 Structure-activity relationships: acyl-CoA thioester substrates AANATL2

Previous data shows that the amine specificity of AANATL2 is dependent on the chain length of the acyl-CoA co-substrate, as the acyl-chain length increased from acetyl-CoA to oleoyl-CoA (Dempsey et al., 2014b). We found that tyramine and octopamine are AANATL2 substrates when acetyl-CoA is the acyl donor; however, tyramine and octopamine will not as substrates when the acyl donor is oleoyl-CoA. Two possible explanations for these data are: (a) longer chain acyl-CoAs will serve as substrates in a different enzyme form that is specific for serotonin or dopamine, or (b) the acyl group of the long-chain acyl-CoAs will occupy the amine binding pocket, resulting in the perturbed and non-productive binding of amine substrates. The $K_{m,app}$ value increased >25-fold for both serotonin and dopamine when a longer chain acyl-CoA (C16 – C20) is used in place of acetyl-CoA. Coupling these data to the dead-end inhibition data of Section 3.2, it is more likely that the longer acyl group is perturbing amine substrate binding, meaning that only certain amines (serotonin and dopamine) will function as substrates when the co-substrate is a long-chain acyl-CoA thioester (Dempsey et al., 2014b).

Overall, the data from the substrate specificity studies reported here and published elsewhere (Dempsey et al., 2014b) show that AANATL2 has significant substrate promiscuity for both acyl-CoA and amine substrates. The three major structural features that affect binding and/or catalysis for AANATL2 substrates were the nature of the amine (arylalkylamine or non-arylalkylamine), the length and α -modification of the spacer in the phenethylamines and the length of the acyl group for the acyl-CoA thioesters. The structure-activity results show that effective AANATL2 catalysis, characterized by a relatively high $(k_{cat}/K_m)_{app}$ value, requires an optimum positioning of the amine for nucleophilic attack at the carbonyl of the acyl-CoA thioester. The ratio of ~10-fold between the $(k_{cat}/K_m)_{app}$ values for the tyramine/octopamine pair and that of ~500 for the benzylamine/phenethylamine pair is consistent with our hypothesis, illustrating that subtle interactions between the substrate and active site amino acids can significantly impact effective catalysis.

3.3 Kinetic mechanism

Defining the kinetic mechanism for AANATL2 could provide insight into the broad specificity for the acyl-CoA substrates and acyl-CoA-dependence of the amine specificity. Using acetyl-CoA and tyramine as substrates, we varied the concentration of one substrate at different, but fixed concentrations of the second substrate. The intersecting double reciprocal plots for both substrates were the outcome of these experiments (Figure 1) strongly

suggesting that AANATL2 will catalyze the formation of *N*-acetyltyramine by a sequential mechanism. This means that a ternary AANATL2•acetyl-CoA•tyramine complex forms prior to catalysis.

Next, we sought to delineate between random or ordered substrate binding for acetyl-CoA and tyramine. We accomplished this using a structural analog for each substrate as an AANATL2 inhibitor. Previous data showed that oleoyl-CoA would not serve as a substrate for AANATL2 when tyramine was the amine substrate (Dempsey et al., 2014b); therefore, we decided to use oleoyl-CoA as an acetyl-CoA analog. Long-chain acyl-CoAs have been used as inhibitors for GNAT enzymes (Dempsey et al., 2014c; Ferry et al., 2000; Khalil and Cole, 1998) and as dead-end inhibitors in elucidating the kinetic mechanism for other *D. melanogaster* AANATL enzymes (Dempsey et al., 2014a, 2015). We employed tyrosol as the other dead-end inhibitor because tyrosol, is an analog of tyramine in which the amino group is replaced with a hydroxyl group. In addition, *O*-acetylation of tyrosol is not catalyzed by AANATL2 because we found no measurable rate of CoA release above background at 1.0 mM acetyl-CoA and concentrations of tyramine as high as 10 mM. Oleoyl-CoA is competitive vs. acetyl-CoA, $K_i = 180 \pm 30$ nM (Figure 2A), and noncompetitive vs. tyramine, $K_i = 540 \pm 40$ nM (Figure 2B). Tyrosol is uncompetitive vs. acetyl-CoA, $K_i = 104 \pm 6$ μ M (Figure 2C), and a competitive vs. tyramine, $K_i = 52 \pm 9$ μ M (Figure 2D). These data indicate that AANATL2 catalyzes the formation of *N*-acetyltyramine via an ordered sequential mechanism, in which acetyl-CoA binds first followed by tyramine to generate the AANATL2•acetyl-CoA•tyramine ternary complex prior to any product formation. An ordered sequential mechanism identified for AANATL2 is consistent with other *D. melanogaster* AANATL enzymes and with the mammalian ortholog, sheep serotonin *N*-acetyltransferase (De Angelis et al., 1998). The fact that the acyl-CoA binds first could provide an explanation for dependence of the amine specificity on the acyl-chain length of the acyl-CoA substrate. Since an AANATL2•acetyl-CoA complex is required prior to tyramine binding, it is likely that the longer-chain the acyl group of oleoyl-CoA can, at least partially, occupy the amine substrate binding site, perturbing the ability for specific amines to bind properly for effective catalysis to occur. This model is supported by the significant increase in the $K_{m,app}$ for serotonin ($7.2 \mu\text{M} \rightarrow 490 \mu\text{M}$) and dopamine ($42 \mu\text{M} \rightarrow 1.2 \text{mM}$), when comparing oleoyl-CoA to acetyl-CoA as the saturating acyl-CoA substrate (Dempsey et al., 2014b). The increase in the $K_{m,app}$ and decrease in the $k_{cat,app}$ values (acetyl-CoA $\rightarrow$ oleoyl-CoA) suggest that as the length of the acyl-CoA aliphatic chain is increased, it begins to occupy the binding site for the amine substrate, leading to non-productive amine binding which results in a decrease in the rate of catalysis.

3.4 AANATL2: acid/base catalysis and the amino acid residues important to this chemistry

We used a series of pH-activity profiles, primary sequence alignments to other *D. melanogaster* AANATL enzymes, and site-directed mutagenesis experiments to generate data that was used to produce a proposed chemical mechanism for AANATL2-mediated catalysis. First, we determined the pH-dependence of the apparent steady-state kinetic constants for acetyl-CoA by varying [acetyl-CoA] at constant [tyramine] as a function of pH (Figure 3). Bell-shaped curves were obtained for both, the $k_{cat,app} - \text{acetyl-CoA}$ and

$(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ – acetyl-CoA profiles yielding two apparent pK_{a} values. The $\text{pK}_{\text{a,app}}$ values from the $k_{\text{cat,app}}$ – acetyl-CoA profile were 7.4 ± 0.2 and 8.9 ± 0.2 , whereas the $\text{pK}_{\text{a,app}}$ values from the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ – acetyl-CoA profile were 6.8 ± 0.2 and 9.0 ± 0.1 . The $\text{pK}_{\text{a,app}}$ values of 7.4 and 6.8 from both profiles, likely represents the same ionizable group - the general base in catalysis. The differences in these two $\text{pK}_{\text{a,app}}$ values most likely results from either the kinetic stickiness of acetyl-CoA or from the difficulty in measuring the acetyl-CoA kinetic constants with varying concentrations that range at the low end of the linear response for the DTNB assay. The second $\text{pK}_{\text{a,app}}$ values of 8.9 and 9.0 represents the same ionizable group, either the general acid in catalysis or the thiol group of coenzyme A with a slightly perturbed pK_{a} . The pK_{a} for the thiol group of coenzyme A has been reported to range from 9.6 to 10.4 (Pitman and Morris, 1980; The Merck Index, 1996).

Studying the pH-dependence of kinetic constants for the AANATL2 wild-type provides information regarding acid/base catalysis, but does not define the specific ionizable groups that account for the measured pK_{app} values. Therefore, we used a combination of primary sequence alignment with other *D. melanogaster* AANATL enzymes (Dempsey et al., 2014a) and site-directed mutagenesis of conserved residues to aid our identification of the respective amino acids that function in catalysis and/or substrate binding. A primary sequence alignment of the eight *D. melanogaster* AANATL enzymes pointed towards five amino acid residues of AANATL2 that could function in catalysis and/or substrate binding: Glu-29, Pro-30, Arg-138, Ser-171, and His-206 (Dempsey et al., 2014b). The amino acids that correlate to these five amino acids from AANATL2 in AANATA (Dempsey et al., 2014a) and AANATL7 (Dempsey et al., 2015) were evaluated by site-directed mutagenesis. Therefore, we can index the data from the site-directed alanine mutants (Table 2) to the corresponding residues from the previously studied *D. melanogaster* AANATL enzymes to delineate a respective function in AANATL2 (Dempsey et al., 2014a, 2015).

In both AANATA and AANATL7, a glutamate has been proposed as the general base in catalysis. The corresponding glutamate in AANATL2 is Glu-29; thus, this amino acid may serve as the general base in catalysis. The $k_{\text{cat,app}}$ value for the E29A mutant was 23% - 25% of the wild-type enzyme, whereas the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ for acetyl-CoA and tyramine decreased 4.3- and 66-fold, respectively (Table 2). The $K_{\text{m,app,acetyl-CoA}}$ for E29A has the same value as wild type AANATL2, indicating that Glu-29 does not function in acetyl-CoA binding. Interestingly, the small decrease in the $k_{\text{cat,app}}$ value suggests that Glu-29 is not the general base in catalysis; however, the large increase in the tyramine $K_{\text{m,app}}$ (17-fold) indicates that this residue is important to amine substrate binding. Since the corresponding glutamates found in *D. melanogaster* AANATA (Glu-47) (Dempsey et al., 2014a) and AANATL7 (Glu-26) (Dempsey et al., 2015), were identified as the general base in catalysis, we wanted to confirm that Glu-29 in AANATL2 does not have the same function in catalysis. This was accomplished by evaluating the pH-dependence on the acetyl-CoA kinetic constants for the E29A mutant (Figure 4). Both, the $k_{\text{cat,app}}$ – acetyl-CoA and $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ – acetyl-CoA profiles for E29A produced a bell-shaped curve with two apparent pK_{a} values, similar to the wild-type. The $\text{pK}_{\text{a,app}}$ values for the E29A mutant were: $k_{\text{cat,app}}$ – acetyl-CoA = 7.0 ± 0.1 and 8.8 ± 0.1 , whereas the $\text{pK}_{\text{a,app}}$ values for the $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ – acetyl-CoA profile = 7.1 ± 0.2 and 9.1 ± 0.2 . The $\text{pK}_{\text{a,app}}$ value of ~ 7.0 for the E29A mutant corresponds to the general base in

catalysis, whereas the $pK_{a,app}$ value of ~ 9.0 is either the general acid in catalysis or the thiol group of the coenzyme A product. The similarity in the $pK_{a,app}$ value of ~ 7.0 for both the E29A mutant and wild type enzymes strongly implies that that Glu-29 is not the general base in AANATL2 catalysis. This is a striking difference from the two other *D.*

melanogaster AANATL enzymes, where the corresponding glutamate functions as the general base (Dempsey et al., 2014a, 2015). When the appropriate glutamate was mutated to an alanine in these enzymes, the pH-rate profile for the alanine mutant has a linear dependence on pH (instead of a bell-shaped profile) with the disappearance of the $pK_{a,app}$ for the general base (Cheng et al., 2012; Dempsey et al., 2014a, 2015) – a very different results than what we found for the E29A mutant. Another possibility to account for our data for the E29A mutant would be that Glu-29 does function as the general base in AANATL2 catalysis, but in a redundant role with another active site amino acid. In this manner, mutation of Glu-29 to Ala would have a relatively minimal effect on catalysis. There is literature precedent for two residues functioning in a redundant role as the general base in catalysis, as observed for sheep serotonin *N*-acetyltransferase (His-120 and His-122) (Hickman et al., 1999a, 1999b; Klein, 2007; Scheibner et al., 2002; Zheng and Cole, 2003). Further evaluation of the redundancy that active site amino acid residues might have with respect to functioning as a general base requires the availability of a crystal structure.

The second $pK_{a,app}$ observed in the wild-type pH-rate profiles ($pK_{a,app} \sim 9.0$) is either the general acid in catalysis or the thiol group of the CoA-SH product. The decrease in the activity at high pH could result from CoA-S⁻ having greater affinity to AANATL2 than the CoA-SH form (K_d , CoA-SH > CoA-S⁻). Two serine residues in AANATA, Ser-182 and Ser-186, were proposed to function in catalysis, with Ser-186 suggested as the general acid (Cheng et al., 2012). In AANATL2, Ser-167 and Ser-171 corresponds to Ser-182 and Ser-186 in AANATA. We prepared both the S167A and S171A mutants and determined the effect of these mutations on catalysis (Table 2). The S167A mutant yielded a $k_{cat,app}$ value that is 47% of the wild-type and a $K_{m,app}$ - acetyl-CoA and $K_{m,app}$ - tyramine that were increased 8- and 4-fold, respectively. The minimal decrease in the S167A $k_{cat,app}$ value suggests that this residue probably does not function directly in catalysis. However, the increase in both substrates $K_{m,app}$ values indicates that this residue has an effect on substrate binding, possibly by functioning in the proper organization of the active site. The second serine mutant, S171A, was completely deficient of catalysis, in which there was not a rate observed above the baseline rate of acetyl-CoA. This could indicate that Ser-171 is the general acid in catalysis or that this residue has a critical function in one (or more) of the following: (a) structural component in the formation of the active site, (b) participates in a “proton wire” by shuttling protons out of the active site (Dempsey et al., 2014a; Hegde et al., 2007; Hickman et al., 1999b; Vetting et al., 2005), or (c) required for the proper positioning of the catalytic residues. Since S171A is catalytically deficient, we could not evaluate the mutant pH-rate profile to determine if this residue corresponds to the second $pK_{a,app}$ value observed in the wild-type enzyme. It seems unlikely that Ser-171 functions as a general acid, since it is thermodynamically unfavorable to transfer a proton from a serine hydroxyl to the thiolate anion of CoA. The serine residue requires a substantial depression in the pK_a value (3-5 pH units) to function in the protonation of a thiolate. It is more likely that this residue has a critical structural function that when disrupted, completely abolishes catalysis.

3.5 Evaluation of AANATL2 amino acid residues that are conserved between other *D. melanogaster* AANATLs

We further evaluated the other conserved residues to understand their respective function in AANATL2. The amino acid residues, Pro-30, Arg-138, and His-220, were each mutated to an alanine and their respective kinetic constants evaluated. To further understand the function of these residues in the absence of a crystal structure, we indexed the data to that generated for the corresponding residue in *D. melanogaster* AANATA (Pro-48) and AANATL7 (Pro-27) (Dempsey et al., 2014a, 2015). Pro-30 corresponds to Pro-48 and Pro-27 in AANATA and AANATL7, respectively. The P30A mutant produced a $k_{cat,app}$ value that is 2% - 3% of the wild-type enzyme, whereas the $K_{m,app}$ for tyramine increased 10-fold. In contrast to the data generated for tyramine, the $K_{m,app}$ for acetyl-CoA was similar to the wild-type enzyme. These data suggest that Pro-30 has an important function in regulating catalysis and the binding of the amine substrate; however, has limited impact on acetyl-CoA binding. The corresponding residue in the mammalian ortholog, sheep serotonin *N*-acetyltransferase (Pro-64) and *D. melanogaster* AANATA is located on a “floppy loop” that occupies the binding site of acetyl-CoA (Dempsey et al., 2014a; Pavlicek et al., 2008). After acetyl-CoA binding, the proline residue migrates $\sim 8 \text{ \AA}$ to open up the amine substrate binding pocket for the sheep ortholog, in addition to having a direct π -stacking interaction with the indole ring of the tryptamine-acetyl-CoA bisubstrate inhibitor. The movement of the “floppy loop” results in the conversion of the enzyme from a low to high affinity state and this particular proline residue is the catalytic switch serving to regulate this conversion (Dempsey et al., 2014a; Pavlicek et al., 2008). The large decrease in the $k_{cat,app}$ value and increase in the $K_{m,app}$ – tyramine suggests, but does not prove, that Pro-30 of AANATL2 is, also, a catalytic switch regulating loop movement in this enzyme, as well. The proposed movement of Pro-30 is important to the formation of the amine substrate binding pocket, providing a structural rationale to the ordered sequential mechanism. Acetyl-CoA binds first, resulting in the Pro-30-dependent movement of the loop to convert AANATL2 from a low affinity state to high affinity state for the amine substrate, followed by the binding of the amine substrate (tyramine, in our specific experiments). This proposed function of Pro-30 as the catalytic switch is consistent with our data and that for the other GNAT enzymes characterized to date (Dempsey et al., 2014a, 2015; Pavlicek et al., 2008). One puzzling aspect in the data for the P30A mutant is lack of difference for the $K_{m,app}$ – acetyl-CoA for the P30A mutant of AANATL2, as was observed for both corresponding proline to alanine mutants constructed for AANATA (Dempsey et al., 2014a) and AANATL7 (Dempsey et al., 2015). The relative position of Pro-30 in the low affinity state does not hinder acetyl-CoA binding for AANATL2 is one possible explanation for our results, as observed for the other AANAT enzymes (Dempsey et al., 2014a; Hickman et al., 1999a; Pavlicek et al., 2008). However, our data for the P30A mutant for *D. melanogaster* AANATL2 clearly shows that Pro-30 does function to regulate catalysis and in the binding of the amine substrate.

Creation of the R138A mutant yielded an enzyme with a $k_{cat,app}$ value that is $\sim 20\%$ of the wild-type enzyme and $K_{m,app}$ values for acetyl-CoA and tyramine that were similar to that of wild-type enzyme. Mutation of the corresponding arginine to alanine in *D. melanogaster* AANATA (Arg-153) and *D. melanogaster* AANATL7 (Arg-138) yielded active mutant enzymes with kinetic constants, $k_{cat,app}$ and the $K_{m,app}$ for both substrates, that were

increased relative to wild-type (Dempsey et al., 2014a, 2015). In AANATA, we proposed that the corresponding arginine, Arg-153, formed a salt bridge with Asp-46 and that the Arg-153:Asp-46 was critical to a conformation responsible for the relatively tight-binding of CoA-S⁻ product. Thus, in AANATA, CoA-SH release is partially rate-determining and elimination of the Arg-153:Asp-46 in the R153A mutant is consistent with the observed increase in $k_{cat,app}$ (Dempsey et al., 2014a). While there is no reported structure for AANATL7, the similarity in the data between AANATA and AANATL7 suggests that Arg-153 in AANATA and Arg-138 in AANATL7 fulfill the same role in both acyltransferases. The differences in the kinetic constants from the R138A mutant in AANATL2 relative to those obtained for the corresponding arginine to alanine mutants in AANATA and AANATL7 indicate that Arg-138 may serve a different role in AANATL2. Without a structure, it is not clear why Arg-138 may have a different function in AANATL2, but some possibilities include: (a) product release might not be rate-limiting for AANATL2 as observed in other GNAT enzymes (Khalil et al, 1998; Farazi et al, 2000; Draker et al, 2003; Vetting et al, 2005), (b) Arg-138 is not within the acyl-CoA binding pocket or in the AANATL2 active site at all, and/or (c) Arg-138 is not found in a salt bridge with any residue in AANATL2. Sequence alignment of AANATL2 to AANATA reveals that the salt bridge partner to Arg-153 in AANATA (Dempsey et al, 2014a), Asp-46, is a glutamine, Gln-28, in AANATL2. A structure of AANATL2 is required to more clearly define the role of Arg-138 in AANATL2 catalysis.

The final AANATL2 amino acid residue evaluated in our study was His-206. The H206A mutant of AANATL2 is inactive with no rate of CoA-SH release above the background acetyl-CoA hydrolysis. The corresponding residues in *D. melanogaster* AANATA (His-220) and AANATL7 (His-206) are important to the active site organization (Dempsey et al., 2014a, 2015). Mutation of both to alanine, H220A and H206A, yields catalytically defective enzymes with $(k_{cat}/K_m)_{app}$ values that are 3%-6% and $k_{cat,app}$ values that are ~22%-30% of wild-type. His-206 may function similarly in AANATL2; however, this residue must be even more critical to active site organization in this enzyme. His-206 could function to organize the active site by positioning a catalytic amino acid residue(s) in the optimal position(s) for catalysis. Also, His-206 could be the general base in AANATL2 catalysis, since our data indicates that Glu-29 cannot serve this role. Again, an AANATL2 structure is required to more definitively define the function of His-206 in AANATL2.

We choose to mutate Glu-29, Pro-30, Arg-138, Ser-167, Ser-171, and His-206 in *D. melanogaster* AANATL2 based on previous research, sequence alignment to related acyltransferases, and structural information from AANATA (Cheng et al., 2012; Dempsey et al., 2014a). Our results for all the AANATL2 amino acids evaluated herein show differences relative to what has been reported for corresponding residues in *D. melanogaster* AANATA and AANATL7. Why are there differences between AANATL2, AANATA, and AANATL7 when the enzymes catalyze a similar reaction with similar substrates (Dempsey et al., 2014a, 2015) – an intriguing question? One possible explanation for the differences is that AANATL2 evolved to have a greater promiscuity for amine or acyl-CoA substrates, especially functioning in the biosynthesis of long-chain *N*-acyldopamines and *N*-acylserotonins (Dempsey et al., 2014b). Could the differences we find for AANATL2

relative to AANATA and AANATL7 be related to structural differences “forced” upon AANATL2 so that this enzyme would accept long-chain acyl-CoA substrates? This question can only be addressed when structures of all the enzymes have been solved.

3.6 Proposed chemical mechanism for AANATL2

Two possible chemical mechanisms consistent with the data reported herein are illustrated in Scheme 2. Currently, our data does not delineate between these two possible mechanisms. The first possible mechanism (Scheme 2A) features: (a) ordered substrate binding to generate the AANATL2•acetyl-CoA•tyramine ternary complex prior to catalysis, (b) deprotonation of the positively charged amine of tyramine by a catalytic base, possibly through ordered water molecules (“proton wire”), (c) nucleophilic attack of the carbonyl of the acetyl-CoA thioester by the deprotonated tyramine to generate the zwitterionic tetrahedral intermediate, and (d) the final breakdown of the zwitterionic tetrahedral intermediate with the concomitant protonation of coenzyme A. The $pK_{a,app}$ values of ~7.0 and 9.0 for this possible mechanism correspond to an undefined general base (His-206?) and the thiolate of CoA-SH. The second possible mechanism (Scheme 2B) consists of: (a) a sequential ordered mechanism with acetyl-CoA binding first followed by tyramine to generate the AANATL2•acetyl-CoA•tyramine ternary complex prior to catalysis, (b) nucleophilic attack of the carbonyl of the acetyl-CoA thioester by tyramine to generate the zwitterionic tetrahedral intermediate, and (c) collapse of the zwitterionic tetrahedral intermediate by a general base catalyzed deprotonation of the positively charged amine intermediate, in addition to a general acid catalyzed protonation of coenzyme A. The $pK_{a,app}$ values of ~7.0 and 9.0 in this possible mechanism corresponds to an undefined general base and general acid in catalysis. Both mechanisms of Scheme 2 are consistent with mechanisms that have been proposed for other GNAT enzymes (Berndsen and Denu, 2008; Farazi et al., 2001; Hegde et al., 2007; Hickman et al., 1999b; Klein, 2007; Thoden et al., 2009; Thoden and Holden, 2010; Vetting et al., 2005) and, in particular, the mechanism depicted in Scheme 2A has been proposed for *D. melanogaster* AANATA and AANATL7 (Dempsey et al., 2014a, 2015). In addition to this chemical mechanism, we highlight that Pro-30, Ser-171, and His-206 are critical in regulating the AANATL2-catalyzed reaction. A structure for AANATL2 is necessary to better define the function of the amino acid residues described herein and to aid in our understanding of its chemical mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work has been supported, in part, by grants from the Florida Center for Excellence for Biomolecular and Targeted Therapeutics (FCoE-BITT grant no. GAL5020), the University of South Florida (a Research Scholarship grant from the College of Arts and Sciences), the Shirley W. and William L. Griffin Foundation, National Institute of Drug Abuse at the National Institutes of Health (R03-DA034323), and National Institute of General Medical Science of the National Institutes of Health (R15-GM107864) to D.J.M., and a Dissertation Completion Fellowship from the Office of Graduate Studies at the University of South Florida to D.R.D.

Abbreviations and Textual Footnotes

AANATL	arylalkylamine <i>N</i> -acetyltransferase
AANATL2	arylalkylamine <i>N</i> -acyltransferase like 2
AANATA	arylalkylamine <i>N</i> -acetyltransferase variant A
AANATL7	arylalkylamine <i>N</i> -acetyltransferase like 7
GNAT	GCN5-related <i>N</i> -acetyltransferase.

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- AANATL2 has promiscuous substrate specificity for acyl-CoA and amine substrates.
- AANATL2 catalyzes the formation of *N*-acetyltyramine through an ordered sequential mechanism.
- Acetyl-CoA binds first, followed by tyramine to generate an AANATL2•acetyl-CoA•tyramine complex prior to catalysis.
- pH-activity profiles highlight an acid/base chemical mechanism with two ionizable groups important to catalysis.
- Functions for certain amino acids residues have been proposed in relation to regulating substrate binding and/or catalysis.

Figure 1.

Double reciprocal plots of initial velocities for acetyl-CoA and tyramine. (A) Initial velocities measured at a fixed concentration of tyramine: 25 μM (●), 10 μM (○), 2 μM (▼), 1 μM (□). (B) Velocities measured at a fixed concentration of acetyl-CoA: 25 μM (●), 10 μM (○), 2 μM (▼), 1 μM (□).

Figure 2.

Dead-end inhibition analysis of AANATL2. (A) Initial velocities measured at a fixed concentration of tyramine (3.9 μM), varying the concentration of acetyl-CoA, and varying the concentration of the inhibitor, oleoyl-CoA: 0 nM (●), 125 nM (○), 250 nM (▼); $K_i = 180 \pm 30$ nM. (B) Initial velocities measured at a fixed concentration of acetyl-CoA (7.2 μM), varying the concentration of tyramine, and varying the concentration of the inhibitor, oleoyl-CoA: 0 nM (●), 125 nM (○), 250 nM (▼); $K_i = 540 \pm 40$ nM. (C) Initial velocities measured at a fixed concentration of tyramine (3.9 μM), varying the concentration of acetyl-CoA, and varying the concentration of the inhibitor, tyrosol: 0 μM (●), 50 μM (○), 100 μM (▼); $K_i = 104 \pm 6$ μM . (D) Initial velocities measured at a fixed concentration of acetyl-CoA (7.2 μM), varying the concentration of tyramine, and varying the concentration of the inhibitor, tyrosol: 0 μM (●), 50 μM (○), 100 μM (▼); $K_i = 52 \pm 9$ μM .

Figure 3. AANATL2 wild-type pH-rate profiles. (A) $k_{cat,app}$ for acetyl-CoA. (B) $(k_{cat}/K_m)_{app}$ for acetyl-CoA.

Figure 4. AANATL2 E29A pH-rate profiles. (A) $k_{cat,app}$ for acetyl-CoA. (B) $(k_{cat}/K_m)_{app}$ for acetyl-CoA.

Scheme 1.

Biosynthetic pathway for the biogenic arylalkylamines and their *N*-acylated products in *D. melanogaster*. (A) Tyrosine biosynthesis. Reaction 1, tyrosine decarboxylase (TDC); 2, tyrosine hydroxylase (TYH); 3, aromatic amino acid decarboxylase (AADC); 4, tyramine β -hydroxylase (T β H); 5, arylalkylamine *N*-acetyltransferase (AANAT). (B) Tryptophan biosynthesis. Reaction 6, tryptophan decarboxylase (TPH); 7, aromatic amino acid decarboxylase (AADC); 8, arylalkylamine *N*-acetyltransferase (AANAT).

Scheme 2.

Proposed chemical mechanisms for *D. melanogaster* AANATL2. R = different acyl chain length for the acyl-CoA thioester.

Table 1Steady-state kinetic constants for the amine substrates^{a,b}

Substrate	Structure	$K_{m, app}$ (μM)	$k_{cat, app}$ (s^{-1})	$(k_{cat}/K_{m})_{app}$ ($M^{-1}s^{-1}$)
3-(Trifluoromethyl)phenethylamine		10 ± 2	4.5 ± 0.2	$(4.4 \pm 0.7) \times 10^5$
4-Methoxyphenethylamine		10 ± 2	3.7 ± 0.1	$(3.7 \pm 0.6) \times 10^5$
3-Methoxyphenethylamine		13 ± 2	4.4 ± 0.2	$(3.5 \pm 0.6) \times 10^5$
5-Benzyloxytryptamine		8 ± 1	2.8 ± 0.1	$(3.3 \pm 0.4) \times 10^5$
Serotonin ^c		7 ± 1	2.4 ± 0.1	$(3.3 \pm 0.1) \times 10^5$
β -Methylphenethylamine		9 ± 1	2.9 ± 0.1	$(3.2 \pm 0.4) \times 10^5$
Tryptamine		14 ± 3	4.3 ± 0.3	$(3.1 \pm 0.6) \times 10^5$
Tyramine		5 ± 1	1.5 ± 0.03	$(2.9 \pm 0.3) \times 10^5$
3,4-Methylenedioxyphenethylamine		27 ± 5	6.3 ± 0.4	$(2.3 \pm 0.4) \times 10^5$

Substrate	Structure	$K_{m, app}$ (μM)	$k_{cat, app}$ (s^{-1})	$(k_{cat}/K_m)_{app}$ ($M^{-1}s^{-1}$)
Phenethylamine		35 ± 10	7.3 ± 1	$(2.1 \pm 0.7) \times 10^5$
Norepinephrine		27 ± 3	5.6 ± 0.3	$(2.1 \pm 0.3) \times 10^5$
Dopamine ^c		42 ± 10	7 ± 1	$(1.6 \pm 0.2) \times 10^5$
4-Phenylbutylamine		9.5 ± 0.4	1.0 ± 0.02	$(1.1 \pm 0.05) \times 10^5$
5-Methoxytyrptamine		25 ± 2	2.6 ± 0.1	$(1.0 \pm 0.1) \times 10^5$
Octopamine ^c		78 ± 4	2.3 ± 0.04	$(3.0 \pm 0.05) \times 10^4$
Benzylamine		210 ± 4	0.092 ± 0.002	430 ± 10
Histamine		3500 ± 200	0.90 ± 0.02	260 ± 5
Ethanolamine		$(1.1 \pm 0.1) \times 10^5$	0.82 ± 0.03	7 ± 1

^aReaction condition – 300 mM Tris pH 8.0, 150 μM DTNB, 50 μM acetyl-CoA, and varying concentration of amine substrate.

^bKinetic constants are reported as $\pm$ standard error (n = 3).

^cData is published in Dempsey et al., 2014b

Table 2Steady-state kinetic constants for AANATL2 mutant enzymes^a

Mutant ^b	Acetyl-CoA			
	K _{m, app} (μM)	k _{cat, app} (s ⁻¹)	(k _{cat} /K _m) _{app} (M ⁻¹ s ⁻¹)	$\frac{\left(\frac{k_{cat}}{K_m}\right)_{app-mutant}}{\left(\frac{k_{cat}}{K_m}\right)_{app-wild-type}} \times 100$
				%
Wild-type	5 ± 1	1.6 ± 0.1	(3.1 ± 0.4) × 10 ⁵	100
E29A	5.1 ± 0.2	0.37 ± 0.01	(7.2 ± 0.3) × 10 ⁴	23
P30A	3.3 ± 0.4	0.032 ± 0.001	(10 ± 1) × 10 ³	3.2
R138A	1.6 ± 0.1	0.313 ± 0.004	(1.9 ± 0.1) × 10 ⁵	61
S167A	40 ± 2	0.75 ± 0.01	(1.9 ± 0.1) × 10 ⁴	6.1
S171A	nd ^c	nd ^c	nd ^c	nd ^c
H206A	nd ^c	nd ^c	nd ^c	nd ^c
Mutant ^d	Tyramine			
	K _{m, app} (μM)	k _{cat, app} (s ⁻¹)	(k _{cat} /K _m) _{app} (M ⁻¹ s ⁻¹)	$\frac{\left(\frac{k_{cat}}{K_m}\right)_{app-mutant}}{\left(\frac{k_{cat}}{K_m}\right)_{app-wild-type}} \times 100$
				%
Wild-type	5 ± 1	1.47 ± 0.03	(2.9 ± 0.3) × 10 ⁵	100
E29A	85 ± 5	0.37 ± 0.01	(4.4 ± 0.3) × 10 ³	1.5
P30A	52 ± 7	0.043 ± 0.001	820 ± 110	0.28
R138A	6 ± 1	0.26 ± 0.01	(4.5 ± 0.7) × 10 ⁴	16
S167A	19 ± 2	1.03 ± 0.04	(5.3 ± 0.7) × 10 ⁴	18
S171A	nd ^c	nd ^c	nd ^c	nd ^c
H206A	nd ^c	nd ^c	nd ^c	nd ^c

^a Kinetic constants are reported as ± standard error (n = 3).^b The reaction rate was measured at a fixed saturating concentration of tyramine while varying the concentration of acetyl-CoA.^c The notation of “nd” is used for a mutant that did not display a rate of coenzyme A release at 412 nm above the baseline hydrolysis rate.^d The reaction rate was measured at a fixed saturating concentration of acetyl-CoA while varying the concentration of tyramine.