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Isolation and characterization of an arylalkylamine *N*-acetyltransferase from *Drosophila melanogaster*

Edith Hintermann[a], Paul Jenö[b], Urs A. Meyer[a,*]

[a]*Department of Pharmacology, Biozentrum of University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland*
[b]*Department of Biochemistry, Biozentrum of University of Basel, CH-4056 Basel, Switzerland*

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Abstract The enzyme arylalkylamine *N*-acetyltransferase (aaNAT) catalyzes the rate-limiting step in melatonin formation in the vertebrate pineal gland. Numerous attempts to purify this highly unstable enzyme from vertebrates have been unsuccessful. Here, we report the purification of an aaNAT enzyme from *Drosophila melanogaster*, using a radioenzymatic activity assay and column chromatography. The isolated 29.5-kDa protein acetylates tryptamine, dopamine and serotonin with affinities of 0.89 to 0.97 mM, respectively. This suggests that the identified aaNAT may be involved in melatonin synthesis and sclerotization as well as in neurotransmitter catabolism in insects.

Key words: Arylalkylamine *N*-acetyltransferase (aaNAT); Melatonin; Pineal gland; Evolution; Invertebrate; *Drosophila melanogaster*

1. Introduction

In the vertebrate pineal gland, an acetyl-CoA-dependent arylalkylamine *N*-acetyltransferase (aaNAT, EC 2.3.1.87) catalyzes the rate-limiting step, the acetylation of serotonin, in the production of melatonin. Melatonin is secreted periodically, resulting in high concentrations at night, translating photoperiodic information via the nervous system into hormonal signals which regulate several physiological processes [1,2]. The circadian rhythm of melatonin production was shown to result from a 10- to 100-fold nocturnal increase in aaNAT activity [3]. The enzyme is hormonally controlled by an endogenous clock located in the hypothalamic suprachiasmatic nucleus (SCN) [4]. At night, the SCN induces the secretion of norepinephrine which acts on the pineal gland through a complex mechanism, involving the production of cAMP as well as Ca^{2+} as second messengers [3]. The final steps of aaNAT activation, as well as the rapid decrease in enzyme activity upon light exposure during or at the end of the dark period, need further studies. Due to the high instability of aaNAT in vertebrates, purification and cloning were unsuccessful so far, preventing studies of aaNAT regulation at the molecular level. Since all organisms are influenced by environmental factors, such as light and darkness, they need a mechanism to coordinate their physiological processes in response to seasonal changes. In various invertebrate species, serotonin, aaNAT activity and melatonin have indeed been found in several organs, including eyes, optic lobe and brain [5–11], supporting the idea that melatonin is an evolutionary highly conserved endogenous mediator of photoperiodic information [5]. In insects, besides melatonin formation (studied e.g. in *Drosophila melanogaster* by Finocchiario et al. [12]), aaNAT plays important roles in the sclerotization of the insect cuticle [13] and the catabolism of neurotransmitters [14–17]. In the present study, we have purified aaNAT from *D. melanogaster*, using tryptamine acetylation as functional enzyme assay. This invertebrate enzyme will hopefully provide the information to identify the corresponding aaNAT in vertebrates.

2. Materials and methods

2.1. Assay for aaNAT activity

A modification of the assay described by Deguchi and Axelrod was used [18]. 50 μl of a diluted protein preparation were mixed with 25 μl tryptamine (Sigma, Germany), 40 mM, in TEN buffer (50 mM Tris-HCl pH 7.2, 1 mM EDTA, 50 mM NaCl) and 25 μl [1-^{14}C]acetyl-CoA (Amersham International, Amersham, UK), 2 mM (specific activity = 0.6 mCi/mmol), in TEN buffer and incubated at 37°C. After 20 min, the incubation was stopped by the addition of 1 ml water-saturated chloroform. The tubes were vortexed (30 s) and centrifuged (12,000 × *g*, 1 min). The aqueous layer was aspirated and the chloroform extract containing the acetylated substrate was washed twice with 200 μl 1 N NaOH. A 500 μl sample of the organic phase was transferred to a scintillation vial and evaporated at 40°C under a stream of nitrogen. 5 ml scintillation cocktail (Emulsifier-Safe, Packard, The Netherlands) were added and radioactivity was determined. If serotonin or dopamine were used as substrate (both Sigma), 40 mM, in TEN buffer, 1 ml isoamylalcohol or toluol/isoamylalcohol (3:2) was added, respectively, to extract the acetylated product [19]. The tubes were vortexed and centrifuged as described above and 500 μl of the organic supernatant were transferred to a scintillation vial and directly mixed with 5 ml scintillation cocktail.

2.2. Maintenance of fly cultures

The Oregon-R wild-type strain of *D. melanogaster* was maintained at 25°C on standard cornmeal agar medium and live yeast, acclimatized to a L12:D12 photoperiod (12 h light, 12 h dark). Adult flies (1–2 days old) were cold-shocked at 4°C, harvested and stored at −70°C until further use.

2.3. Enzyme purification

Flies were ground in a mortar at a concentration of 1 g/3 ml in ice-cold TEND buffer (TEN buffer with 2 mM DTT (dithiothreitol)), containing 10% glycerol, 0.1 mM PMSF (phenyl-methyl-sulfonyl-fluorid), 0.02 mM BHT (butylated hydroxylated toluene), 0.01 mM pepstatin and 0.1 mM trypsin inhibitor. The homogenate was centrifuged at 4°C for 30 min at 10,000 × *g*. The supernatant was filtered through Miracloth (Calbiochem, San Diego, CA) and recentrifuged. A 100-ml aliquot of this supernatant fraction was brought to 15% saturation with a stock solution of 60% PEG (polyethyleneglycol) 8000 in TEN. After 1 h incubation at 4°C on a magnetic stirrer, the sample was centrifuged for 30 min at 10,000 × *g*. The supernatant was diluted 1:5 with TEND buffer for application to a column (4.5 cm × 5 cm) of DEAE-Sepharose (Pharmacia, Uppsala, Sweden), equilibrated with 3% PEG 8000 in TEN at a flow rate of 8.5 ml/min. After stepwise washing with 7 column volumes of TEN and 4 column volumes of TEN containing 80 mM NaCl, enzyme activity was eluted with TEND containing 170 mM NaCl (total volume 160 ml). The active pool was concentrated to 2 ml by ultrafiltration through a centriprep10 membrane (Amicon,

*Corresponding author. Fax (41) (61) 267 22 08.

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Grace company, Beverly, MA). The sample was loaded onto a Sephacryl S-200 (Pharmacia) column (1.5 cm × 80 cm) which was developed at 10 ml/h with 5 mM sodium phosphate buffer (pH 6.8) containing 2 mM DTT. Active fractions (2 ml) were pooled and applied to a Bio-Gel HTP Hydroxylapatite (BioRad, Richmond, CA) column (1.5 cm × 4.5 cm) at a flow rate of 35 ml/h. The column was washed with 20 ml 5 mM sodium phosphate (pH 6.8), collecting 4-ml fractions. Proteins were eluted with a linear gradient (100 ml) from 5 to 50 mM phosphate in the same buffer and in addition a final wash was performed with 20 ml 55 mM phosphate buffer. The pool of active fractions (54 ml) was brought to a 1.7 M ammonium sulfate concentration by adding solid ammonium sulfate. The sample was loaded on a Butyl-Sepharose (Pharmacia) column (1.5 cm × 1.5 cm), equilibrated with 1.7 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 6.8) at a flow rate of 10 ml/h. After washing with 12 ml equilibration buffer, the proteins were eluted with 20 ml 1.3 M ammonium sulfate in 50 mM phosphate buffer (pH 6.8), collecting 1-ml fractions. Fractions 7–18 were pooled and TCA (trichloracetic acid) precipitated. The pellet was stored at −70°C.

Protein content was determined with a dye-binding assay, using bovine serum albumin as standard [20].

2.4. Internal peptide sequence determination

The collected TCA pellets of aaNAT were subjected to two-dimensional gel electrophoresis as described by O'Farell [21]. The TCA pellets were resuspended in sample buffer containing 2.0% nonidet P40 and focused using a pH gradient from pH 3.0 to pH 10 (1.6% 5/7 ampholyte and 0.4% 3/10 ampholyte; BioRad). The second dimension was SDS-PAGE with a 15% polyacrylamide gel which was Coomassie blue-stained for protein detection. The protein spot containing aaNAT was digested in the gel piece with endoproteinase LysC (Wako Chemicals, Neuss, Germany). Peptide fragments were separated by reverse-phase HPLC and selected fractions were subjected to automated Edman degradation as described in [22].

2.5. Determination of pI and M_r

The isoelectric point (pI) was determined with a pI marker kit (BioRad) under the same conditions as used before protease digest of aaNAT in the gel piece (see above). M_r was estimated by SDS-PAGE, using appropriate low molecular weight standard proteins (BioRad).

2.6. Kinetic studies

Velocities of amine acetylation were determined by keeping the concentration of [1-^{14}C]acetyl-CoA at 2 mM and varying substrate concentrations from 0.1 to 16 mM for tryptamine, from 0.02 to 2 mM for serotonin and from 0.005 to 4 mM for dopamine. K_m was determined by the data analysis program Grafit [23].

3. Results and discussion

Since the vertebrate aaNAT is highly unstable during purification and distinct from already characterized *N*-acetyltransferases [24], protein isolation or homology-based cloning of aaNAT were without success so far. The assumptions that an evolutionary highly conserved molecule, like melatonin, is formed by an evenly conserved biosynthetic pathway and that the enzyme might be more stable in a simpler organism led us to isolate and characterize aaNAT from an invertebrate species. aaNAT activity in *D. melanogaster* was indeed relatively stable; enzyme activity in a sample of the first chromatographic step (DEAE-Sepharose) was only slightly reduced to 84% of the initial activity when incubated for 3 days at 4°C. Thus, using tryptamine acetylation as functional enzyme assay, the purification of a protein fraction with high arylalkylamine *N*-acetyltransferase activity to apparent electrophoretic homogeneity was achieved. The described isolation procedure resulted in a 490-fold purification with a recovery of 7.4% (Table 1). A 15% polyethyleneglycol precipitation was followed by classical column chromatography. The last chromatography step, hydrophobic interaction chromatography on a Butyl-Sepharose column, led to a protein elution pattern with one single band whose staining intensity correlated with the enzyme activity measured in the corresponding fractions (Fig. 1). The isolated enzyme showed an apparent molecular mass of 29.5 ± 0.1 kDa as estimated by SDS-PAGE (n = 3, values = mean ± S.D.).

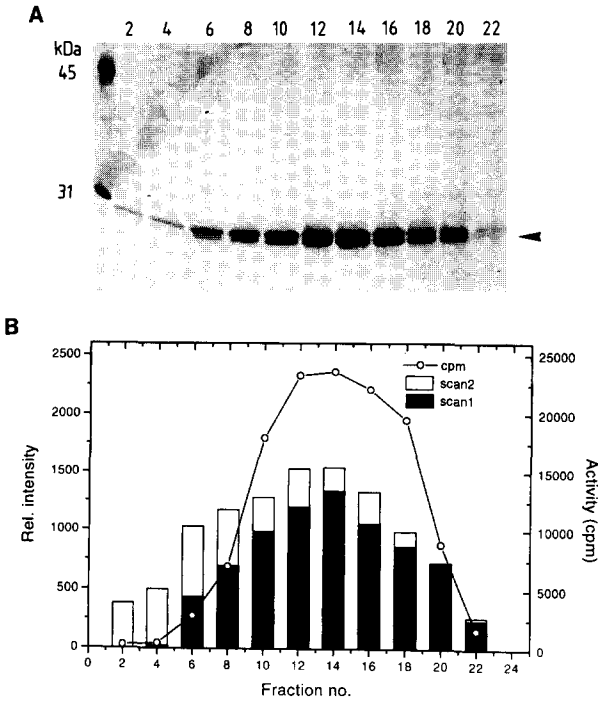

Fig. 1. Identification of aaNAT protein after hydrophobic interaction chromatography. After Butyl-Sepharose chromatography, eluted fractions were analysed for protein content on a silver stained 15% acrylamide gel (A) and for aaNAT enzyme activity. (B) The relative band intensities were determined by densitometry: Scan1 = relative intensity of the marked lower band (arrow head); scan2 = relative intensity of the major contaminating band. Band intensities were compared with the measured enzyme activity (curve, in cpm).

Table 1
Purification of arylalkylamine *N*-acetyltransferase from *D. melanogaster*

Step	Protein (mg)	Total activity (μmol/h)	Spec. activity (μmol/mg × h)	Purification (fold)	Recovery (% of total activity)
10,000 × *g* supernatant	560	1974	3.5	–	–
PEG supernatant	91.8	1244	13.5	3.8	63
DEAE-Sepharose	4	827	206.5	58.6	42
Centriprep	3	789.5	268.5	76.2	40
Sephacryl S-200	1.5	522	348	98.7	26.5
Hydroxylapatite	0.38	384	1010	288.5	19.5
Butyl-Sepharose	0.085	146	1715	490	7.4

Table 2
Affinities of aaNAT for different substrates after Hydroxylapatite chromatography

Substrate	Michaelis-Menten constant (K_m (mean ± S.D.))
Tryptamine	0.89 ± 0.04 mM (n = 5)
Serotonin	0.91 ± 0.39 mM (n = 4)
Dopamine	0.97 ± 0.30 mM (n = 4)

This value corresponds to studies by Maranda and Hodgetts [25], who determined native aaNAT of *D. melanogaster* as a 29-kDa enzyme, indicating that aaNAT probably is a monomer. Using a pI marker kit, the isoelectric point was determined as 5.52 ± 0.02 (n = 4, values = mean ± S.D.). To reach a high purity of the isolated protein, the pool of active fractions after hydrophobic interacion chromatography was subjected to two-dimensional gel electrophoresis prior to further analysis of aaNAT by internal sequence determination. Assuming a mean molecular mass of 29 kDa, 16% of the total amino acid sequence could be determined (Table 2) by automated N-terminal sequencing. The peptides showed neither evident homology to other *N*-acetyltransferases nor to *D. melanogaster* proteins in a SWISSPROT databank search. Thus, the isolated protein is yet unidentified and might belong to a new subfamily of *N*-acetyltransferases.

Acetylation of tryptamine may represent the involvement of aaNAT in neurotransmitter inactivation. In addition, amine acetylation plays a role in melatonin synthesis with serotonin as substrate and the hardening of the insect cuticle involves the acetylation of dopamine. We, therefore, were interested in the affinities of aaNAT for the three different substrates. K_m values for tryptamine, serotonin and dopamine were determined in the pool of active fractions after Hydroxylapatite chromatography. As shown in Table 3, aaNAT acetylates all tested amines with the same affinity, indicating that the purified enzyme might be involved in all three acetylation functions.

If light-regulated melatonin formation is indeed a general process in all animals, an important question is if the characteristics of the *Drosophila* aaNAT protein are comparable to the properties of the pineal aaNAT. A first criterion were our kinetic studies: the K_m value of 0.89 mM for tryptamine acetylation in *Drosophila* lies in the same order of magnitude as the K_m of 0.16 mM found for tryptamine acetylation in the pineal gland of rabbits [24]. Further parallels were found in the behavior during purification: the isolated protein had a molecular mass of around 30 kDa. In forskolin/camptothecin-stimulated Y79 human retinoblastoma cells, a 30-kDa protein was recently identified by Janavs et al. [26], the radiolabeling of which was consistently increased with increases in aaNAT activity and melatonin concentration. In addition, as in protocols for the isolation of pineal aaNAT, the presence of the reducing agent DTT was required in order to prevent loss of enzyme actvitiy.

Table 3
Internal peptide sequences of aaNAT

4aa	Tyr-Ser-Leu-Lys
5aa	Thr-Phe-Phe-Phe-Lys
14aa	Asp-Glu-Pro-Leu-Asn-Thr-Phe-Leu-Asp-Leu-Gly-Glu-Cys-Lys
20aa	Val-Asp-Gln-Asp-Cys-Pro-Tyr-Thr-Ile-Glu-Leu-Ile-Gln-Pro-Glu-Asp-Gly-Gly/Glu-Ala-Val

The last step in melatonin synthesis is the methylation of acetylserotonin by the enzyme hydroxyindole-*O*-methyltransferase (HIOMT). Since human pineal HIOMT exhibits homology with a plant and four bacterial *O*-methyltransferases [27] and antibodies raised against bovine pineal HIOMT cross-react with cockroach cells in the optic lobe [28], the melatonin synthesis pathway seems indeed highly conserved in evolution. The *Drosophila* aaNAT cDNA could, therefore, become an important tool for the isolation of the pineal aaNAT. The molecular cloning of this cDNA is our next goal.

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References

[1] Reiter, R.J. (1980) Endocr. Rev. 1, 109–131.
[2] Reiter, R.J. (1991) Endocr. Rev. 12, 151–180.
[3] Klein, D.C., Schaad, N.L., Namboordiri, M.A.A., Yu, L. and Weller, J.L. (1992) Biol. Soc. Trans. 20, 299–304.
[4] Klein, D.C., Weller, J.L. and Moore, R.Y. (1971) Proc. Natl. Acad. Sci. USA 68, 3107–3110.
[5] Vivien-Roels, B. and Pévet, P. (1993) Experientia 49, 642–647.
[6] Evans, P.H., Soderland, D.M. and Aldrich, J.R. (1980) Insect Biochem. 10, 375–380.
[7] Shampengtong, L., Wong, K.P. and Ho, B.C. (1987) Insect Biochem. 17, 111–116.
[8] Martin, R.J., Jahagirdar, A.P. and Downer, R.G.H. (1989) Insect Biochem. 19, 351–359.
[9] Kempton, L.R.C., Isaac, R.E., Pillmoor, J.B. and Willis, R.J. (1992) Insect Biochem. Mol. Biol. 22, 777–783.
[10] Whitaker, D.P. and Goosey, M.W. (1993) Biochem. J. 295, 149–154.
[11] Withyachumnarnkul, B., Buppaniroj, K. and Pongsa-Asawapaiboon, A. (1992) Comp. Biochem. Physiol. 102A, 703–707.
[12] Finocchiario, L., Callebert, J., Launay, J.M. and Jallon, J.M. (1988) J. Neurochem. 50, 382–387.
[13] Karlson, P. and Sekeris, C.E. (1962) Nature (London) 195, 183–184.
[14] Sekeris, C.E. and Karlson, P. (1966) Pharmacol. Rev. 18, 89–94.
[15] Dewhurst, S.A., Croker, S.G., Ikeda, K. and McCaman (1972) Comp. Biochem. Physiol. 43B, 975–981.
[16] Evans, P.H. and Fox, P.M. (1975) J. Insect. Physiol. 21, 343–353.
[17] Nishimura, K., Fujita, T. and Nakajima, M. (1975) Pest. Biochem. 5, 557–565.
[18] Deguchi, T. and Axelrod, J. (1972) Anal. Biochem. 50, 174–179.
[19] Deguchi, T. (1975) J. Neurochem. 24, 1083–1085.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] O'Farell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[22] Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990–7997.
[23] Leatherbarrow, R.J. (1992) GraFit Version 3.0. Erithacus Software.
[24] Heim, M.H., Blum, M., Beer, M. and Meyer, U.A. (1991) J. Neurochem. 57, 1095–1099.
[25] Maranda, B. and Hodgetts, R. (1977) Insect. Biochem. 7, 33–43.
[26] Janavs, J.L., Florez, J.C., Pierce, M.E. and Takahashi J.S. (1995) J. Neurosci. 15, 298–309.
[27] Donohue, S.J., Roseboom, P.H., Illnerova, H., Weller, J.L. and Klein, D.C. (1993) DNA Cell Biol. 12, 715–727.
[28] Takeda, M., Endo, Y., Saito, H., Nishimura, M. and Nishiitsutsuji-Uwo, J. (1985) Biomed. Res. 6, 395–400.