Purification and properties of the enzyme arylamine *N*-acetyltransferase from the housefly *Musca domestica*

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The enzyme arylamine *N*-acetyltransferase (ANAT) from the housefly (*Musca domestica*) has been purified. The M_r of the purified enzyme was $27\,600 \pm 1\,700$ as estimated by gel filtration. SDS/PAGE yielded a value of $26\,000 \pm 300$, clearly indicating a monomeric structure. The purified enzyme had apparent K_m values for acetyl-CoA and tyramine of $8.4 \mu\text{M}$ and $8.8 \mu\text{M}$ respectively, a pH optimum of 7.2 in 10 mM potassium phosphate buffer and an apparent pI of 5.8. ANAT activity showed a strong dependency on the presence of 2-mercaptoethanol during the

purification stages. The enzyme could be completely inactivated by treatment with *p*-chloromercuribenzoate although the enzyme activity was protected by preincubation with acetyl-CoA. One or more cysteine residues are clearly required for catalytic activity, as demonstrated for the mammalian enzyme. In contrast, partial sequencing of the enzyme has yielded a number of peptide sequences, including the N-terminal sequence, which show no similarity with those reported for the mammalian and avian enzymes.

INTRODUCTION

The enzyme arylamine *N*-acetyltransferase (E.C. 2.3.1.5) catalyses the acetylation of a wide range of aromatic amines using acetyl-CoA as acetyl donor. In mammals the enzyme is involved in generating melatonin from 5-hydroxytryptamine (5-HT; serotonin) in the pituitary gland (Weissbach et al., 1960) and as a detoxification mechanism for xenobiotics in the liver, e.g. in the metabolism of hydrazine and arylamine drugs (Weber, 1973). The pineal 5-HT *N*-acetyltransferase is distinct from the liver xenobiotic enzyme. The latter enzyme has been purified and characterized from pigeon (Andres et al., 1983), rabbit (Andres et al., 1987), chicken (Deguchi et al., 1988) and rat (Yu and Boulton, 1979). The enzyme from chicken liver has been partially sequenced and the cDNA has been cloned and expressed in Chinese hamster ovary cells (Ohsako et al., 1988). These studies have led to the isolation of several cDNAs from other mammalian species, including rabbit (Blum et al., 1989) and human liver (Ohsako and Deguchi, 1990).

In insects, the enzyme was shown to be responsible for the formation of *N*-acetyldopamine, an important component in the sclerotization of the insect cuticle (Karlson and Sekeris, 1962), in puparium formation (Sekeris, 1964) and in the tanning of oviposited eggs (Li and Nappi, 1992). In addition to its role in the sclerotization process, Sekeris and Karlson (1966) first suggested that ANAT may be important in regulating the metabolism of neurogenic catecholamines. It was not until the work of Dewhurst et al. (1972) that the enzyme was demonstrated to metabolize biogenic amines in insect nervous tissue, in an analogous role to monoamine oxidase and catecholamine *O*-methyltransferase in the inactivation of catecholamines in the mammalian nervous system. This study demonstrated that *N*-acetylation was probably the major route for biogenic amine metabolism in *Drosophila* nervous tissue.

Several studies have confirmed and extended these observations by indicating the primary importance of ANAT in metabolizing 5-HT in the honey-bee, *Apis mellifera*, brain (Evans and Fox, 1975), tryptamine metabolism in cockroach, *Periplaneta americana*, heads (Nishimura et al., 1975) and octopamine

metabolism in *Locusta migratoria* (Hayashi et al., 1977) and cwnattle tick, *Boophilus microplus* (Kempton et al., 1992) nerve tissue. Despite the importance of ANAT in the inactivation of biogenic amines such as octopamine in the insect nervous system, the enzyme has only been characterized from crude or partially purified enzyme preparations (e.g. Maranda and Hodgetts, 1977; Evans et al., 1980; Shampengtong et al., 1987; Martin et al., 1989).

In this paper we report a rapid purification of the enzyme from the housefly, *Musca domestica*. Some chemical characteristics, including the partial amino acid sequence, are described and compared with those reported from mammalian and insect sources.

EXPERIMENTAL

Materials

Acetyl-CoA (sodium salt), tyramine, 2-mercaptoethanol, SDS, Coomassie Blue R-250, Tris buffer, Bis-Tris buffer and amethopterin were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Sephadex, Sepharose, Blue Sepharose and other f.p.l.c. and Phastsystem supplies were from Pharmacia Biotechnology, Milton Keynes, Bucks., U.K. *N*-acetyltyramine was synthesized at Sittingbourne Research Centre by the method described by Hayashi et al. (1977). Immobilon P was from Millipore, Watford, Herts., U.K., and [7-¹⁴C]tyramine (specific radioactivity 2.07 GBq/mmol) from Amersham International, Amersham, Bucks., U.K. Ultrafiltration membranes were from Amicon. Hydropore AX was purchased ready-packed from Rainin Instruments, Emeryville, CA, U.S.A. Optiphase scintillation fluid was from LKB Instruments. Other chemicals of analytical reagent grade were obtained from BDH Chemicals, Poole, Dorset, U.K.

Maintenance of housefly cultures

Larvae of *M. domestica* were fed on a diet of baker's yeast/milk/bran and adults on a diet of 50 % (w/v) milk powder in water and maintained at 25 °C in an 8 h light and 16 h dark

cycle. Adult flies were harvested when about 3 days old and immediately frozen at -80°C until required.

Isolation of insect material

The body parts, heads, thoraxes and abdomens, were separated by sieving the frozen whole insects through a sieve pile (2.36 mm, 2.0 mm, 1.7 mm, 1.4 mm, 1.0 mm) on a shaker, resulting in the collection of fairly pure preparations of separate body parts. The major source of ANAT activity was detected in the head fraction (results not shown); however, extracts of head tissue contained large quantities of eye pigment which interfered with subsequent protein purification, so the thorax fraction was used for enzyme purification.

Enzyme assay

Enzyme activity was measured using a radiochemical assay based on the conversion of [^{14}C]tyramine into *N*-acetyl[^{14}C]tyramine (Dewhurst et al., 1972). A typical reaction mixture contained 5 μmol of potassium phosphate, pH 7.0, 6 nmol of acetyl-CoA (sodium salt), and 45 nmol of [7- ^{14}C]tyramine hydrochloride (specific radioactivity 0.825 $\mu\text{Ci}/\mu\text{mol}$) in a total volume of 0.3 ml. Assays were performed at 37°C for 15 min in polypropylene 1.5 ml microfuge tubes. The mixture was equilibrated for 1 min and the reaction started by the addition of radiolabelled tyramine and stopped by the addition of 1 ml of ice-cold ethyl acetate and shaken immediately. The tubes were mixed continuously for 30 min on a vortex shaker before the organic and aqueous phases were separated by centrifugation in a microfuge for 5 min. A sample (0.5 ml) of the ethyl acetate layer was added to 5 ml of Optiphase scintillation fluid and radioactivity determined in a liquid scintillation counter (LKB, 1209 Rackbeta).

The identity of the radioactive product formed was checked by t.l.c. on a Berthold LB 7223 plate scanner (solvent system was butanol/acetic acid/water, 4:1:1 by vol.). A sample of the ethyl acetate fraction showed a single radioactive peak which corresponded to that of authentic *N*-acetyltyramine (results not shown).

Enzyme activity was determined from gradient (8–25%) polyacrylamide gels by cutting the gels into 0.35 cm strips and adding each strip to a standard assay mixture as described above, followed by a 30 min assay period.

One enzyme unit was defined as the amount of enzyme required to catalyse the formation of 1 μmol of acetyltyramine/min at pH 7.0 and 37°C .

Chemical inhibition studies

A sample (5 μg) of a partially purified preparation of ANAT (post-Hydropore AX step) was incubated for 5 min with 0.033 μM –3.3 μM *p*-chloromercuribenzoate (PCMB), at 37°C in 10 mM potassium phosphate buffer, pH 7.0, in the presence or absence of 20 μM acetyl-CoA. The reaction was started by the addition of [^{14}C]tyramine and enzyme activity determined after 10 min as described previously.

Protein determination

Protein content was determined using the Pierce bicinchoninic acid method for crude samples and the Bradford dye-binding method (Bradford, 1976) and A_{280} measurements for relatively pure samples containing small amounts of protein. BSA was used as a standard.

Purification of ANAT

Preparation of insect material

Frozen fly thoraxes (100 g) were homogenized in 300 ml of buffer (50 mM Hepes, pH 7.0/1 mM 2-mercaptoethanol containing 10 μM phenylmethanesulphonyl fluoride) at room temperature, in a Waring blender at full speed for 1 min. The crude homogenate was filtered through four layers of muslin into an ice-cold vessel and the filtrate centrifuged at 20000 g for 45 min. The supernatant was filtered through a glass wool plug to remove any lipid and subsequently used for further purification.

Protein from the supernatant was precipitated with solid ammonium sulphate and the 50–65% (w/v) ammonium sulphate fraction was collected by centrifugation at 20000 g for 10 min. The pellet was redissolved in a small volume of buffer (5 mM Hepes, pH 7.2/1 mM 2-mercaptoethanol) and desalted on a Sephadex G25 column (2.5 cm $\times$ 17 cm). At this stage it was often convenient to store the preparation overnight, and this could be done without loss of activity at 4°C . All subsequent operations were performed at 4°C except for the f.p.l.c. steps which were performed at room temperature.

Affinity chromatography on Blue Sepharose CL-6B

The desalted extract was applied in a volume of 10–30 ml to a column (2.5 cm $\times$ 20 cm) of Blue Sepharose CL-6B, pre-equilibrated in buffer (5 mM Hepes, pH 7.0/1 mM 2-mercaptoethanol) and allowed to equilibrate, by recycling the eluate, for about 3 h at a flow rate of 1 ml/min. The column was then washed with buffer followed by a step-wise elution using buffer containing 0.5 M (100 ml) and then 1 M (50 ml) NaCl. The eluted enzyme was concentrated to 2 ml in an Amicon ultrafiltration cell fitted with a PM10 membrane and then desalted in 1 ml batches on a Pharmacia Fast Desalting column.

F.p.l.c. procedures

Chromatography columns were pre-equilibrated for about 1 h before use and all buffers were made up in Milli Q deionized water. Protein samples were filtered through sterile 0.2 μm -pore-size Millipore filters before injection on to a column. All fractions were maintained at 4°C , immediately after elution, before assaying for ANAT activity.

Ion-exchange chromatography

The desalted preparation was applied in a volume of 10 ml to a Hydropore AX column (Rainin, 10 cm $\times$ 1 cm) fitted to an f.p.l.c. system. The column was equilibrated with 5 mM Hepes (pH 7.2) containing 1 mM 2-mercaptoethanol, at a flow rate of 0.5 ml/min. The enzyme was loaded on to the column, allowed to equilibrate and eluted in a linear gradient of 0–0.5 M NaCl in buffer (total volume 20 ml). Fractions (1 ml) were collected and those with the highest specific activity (14–16) were pooled for further purification.

Chromatofocusing

The pooled fraction from the ion-exchange column was applied, in a volume of 10 ml, to a Mono P column (HR 5/20). The column was washed with 25 mM Bis-Tris buffer (pH 7.0) at a flow rate of 0.5 ml/min, after equilibration, a shallow pH gradient (pH 4.0–7.0) was developed using Polybuffer 74 (30 ml) and 1.0 ml fractions were collected. The pH gradient was monitored by a flow-through pH meter. Fractions containing the highest

Figure 1 Chromatofocusing of pooled fractions from Hydropore AX chromatography

Conditions are described in the Experimental section.

enzyme activity (16–18) were pooled (Figure 1) and concentrated to a small volume (about 0.5 ml) by ultrafiltration in an Amicon 'centricon'.

Gel filtration

Final purification was carried out on a Superose 12 f.p.l.c. column equilibrated in 5 mM potassium phosphate (pH 7.0) containing 1 mM 2-mercaptoethanol. Samples were loaded in a volume of 0.1–0.2 ml and the column developed at a flow rate of 1.0 ml/min with the equilibration buffer. Fractions (1 ml) were collected and those containing the highest enzyme activity (14–16; Figure 2a) were pooled and stored at -20°C .

PAGE

SDS/PAGE was carried out in an LKB vertical slab gel apparatus or using a Pharmacia Phastsystem. For the LKB system, gels were prepared by the method of Weber and Osborne (1969) using the Tris–glycine buffer system. Protein samples were denatured by heating at 90°C for 5 min in the presence of a mixture of 10 mM 2-mercaptoethanol and 1% (w/v) SDS. A $20\ \mu\text{l}$ sample was applied to the gel and electrophoresis was performed at a constant current of 25 mA for 90 min. The gels were stained with Coomassie Blue R-250 for 2 h before destaining in methanol/acetic acid/water (1:1:8, by vol.). For the Pharmacia Phastsystem, electrophoresis was carried out on 8–25% gels using 0.55% SDS buffer strips and run at 65 V·h. Proteins were identified by silver staining.

Native PAGE was carried out on a Phastsystem using 8–25% gradient gels and run at 260 V·h with protein detected by silver staining and by large (20 cm $\times$ 20 cm) homogeneous gels (8%) run on an LKB vertical electrophoresis system for 2 h at 30 mA and protein detected by Coomassie Blue staining.

Determination of M_r

The apparent M_r of the native protein was determined using gel filtration on a Superose 12 column (f.p.l.c.) calibrated with a standard gel filtration calibration kit (Pharmacia). The column was developed at a flow rate of 1 ml/min in 5 mM Tris, pH 7.0/0.15 M NaCl at room temperature.

Figure 2 Superose 12 chromatography of pooled fractions obtained from chromatofocusing chromatography

(a) Conditions are described in the Experimental section. (b) Calculation of ANAT M_r on a Superose 12 column. Conditions are described in the Experimental section.

Denaturing and native electrophoresis were performed using a Pharmacia Phastsystem as previously described and calibrated with the following M_r markers: cytochrome *c* (12 300), myoglobin

Table 1 Summary of purification of ANAT from a crude brei of *M. domestica* thoraxes

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude brei	300	8700	926	0.11	1	100
Desalted (NH ₄) ₂ SO ₄ fraction (50–65%)	30	1287	903	0.70	6	98
Blue Sepharose	44	25.9	849	33	300	92
Hydropore Ax f.p.l.c.	3	1.88	403	214	1945	44
Chromatofocusing f.p.l.c.	8	0.134	331	2470	22 454	36
Superose 12 f.p.l.c.	6	0.054	162	3000	27 273	17

(17200), carbonic anhydrase (30000), ovalbumin (42700), BSA (66200) and ovotransferrin (77000).

Amino acid sequencing

N-terminal sequencing was carried out on approx. 1 nmol of protein attached to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon P). The protein was transferred from a polyacrylamide gel on to a PVDF membrane using a Millipore Milliblot SDE semi-dry system. Internal sequencing of purified ANAT was determined on peptide fragments derived from tryptic digests [2% (w/v) trypsin in 100 mM ammonium carbonate for 6 h at 37 °C]. Separation of the derived peptides was accomplished by reversed-phase h.p.l.c., using a C₈ column (2 mm × 100 mm) with a linear gradient of 0.08% trifluoroacetic acid (TFA) to 0.08% TFA and 90% acetonitrile at a flow rate of 0.2 ml/min and u.v. detection at 214 nm.

Protein sequencing was carried out by Jaytee Biosciences (Kent R&D Centre, University of Kent, Canterbury, Kent, U.K.) using a solid-phase Edman degradation system.

RESULTS

Purification and molecular properties

The purification steps of a typical experiment are summarized in Table 1. This scheme resulted in the purification of ANAT to apparent homogeneity, as determined by several analytical measurements: on native PAGE, silver staining revealed only one protein band (Figure 3), enzyme activity could be recovered from a similar section of a native polyacrylamide gel (Figure 4)

Figure 3 SDS/PAGE of *M. domestica* ANAT

The gel was stained with silver stain and scanned on a recording densitometer. Standard *M_r* proteins were used as described in the Experimental section.

and N-terminal analysis showed only a single amino acid sequence (Table 2).

On one occasion, a large-scale preparation was carried out using approx. 1 kg of fly thoraxes, but it was found that a number of the procedures, principally the Blue Sepharose CL-6B step, were not suitable as large-scale techniques. The relatively small-scale procedure using approx. 100 g of insect thoraxes, was, however, reproducible and yielded consistently 30–100 µg of homogeneous protein within 4 days. This procedure was repeated in 12 different experiments. However, on two occasions, the enzyme activity eluting from the chromatofocusing step was found in two close, but discernible, peaks at a pI of 5.85 and 5.80 respectively (results not shown). On all other occasions, the enzyme eluted as a single peak with an apparent pI value of 5.80.

Gel-filtration experiments with ANAT on a calibrated Superose 12 column gave an *M_r* of 27600 ± 1700 (*n* = 2). SDS/PAGE of the purified enzyme routinely yielded a single protein band, indicating a subunit *M_r* of 26000 ± 300 (*n* = 3). In addition ANAT activity recovered from a native gradient polyacrylamide gel (see Figure 4) was located only in the region between the two *M_r* marker proteins, cytochrome *c* (12300) and carbonic anhydrase (30000).

Enzyme stability

The enzyme isolated from *M. domestica* was quite stable. Partially purified preparations were stored at –20 °C for 6 months without any apparent loss of enzyme activity. The homogeneous enzyme was less stable than partially purified material and repeated freeze/thawing resulted in loss of catalytic activity. For long-term storage, the homogeneous enzyme was stored in small aliquots at –20 °C. Addition of 2-mercaptoethanol to the buffers used in the purification steps was found to be essential for maintaining an active enzyme preparation.

pH optimum

The pH optimum for the enzyme in 10 mM potassium phosphate buffer is fairly broad over the range pH 6.5–8.0, with an optimum at pH 7.2 (results not shown).

Kinetic characteristics

All kinetic measurements were carried out using a partially purified preparation of ANAT (post-Hydropore AX step). The apparent *K_m* value for tyramine in potassium phosphate buffer at pH 7.0 containing 20 µM acetyl-CoA was 8.8 µM. Similarly, the apparent *K_m* value for acetyl-CoA was 8.4 µM in the presence of 200 µM tyramine.

Figure 4 ANAT activity as measured after electrophoresis on a native gradient (8–25%) polyacrylamide gel

The gel was cut into 0.35 mm strips and enzyme activity determined as described in the Experimental section. The standard proteins shown are carbonic anhydrase (M_r 31 000) and cytochrome *c* (M_r 12 300).

Table 2 N-terminal and internal amino acid sequences for ANAT from *M. domestica*

Xaa represents those amino acids that could not be unambiguously assigned.

Sequence	Composition of sequence
1 (N-terminus)	Met-Ile-Val-Ala-Val-Phe-Ala-Ile-Xaa-Gln-Phe-Asn
2 (tryptic peptide)	Ser-Pro-Glu-Val-Asp-Gln-Phe
3 (tryptic peptide)	Ala-Ile-Pro-Ala-Val-Tyr-Ala-Ile
4 (tryptic peptide)	Leu-Gly-Xaa-Xaa-Asn-Ile-Xaa-Val-Val-Xaa-Ser-Xaa-Xaa-Gln

Table 3 Inhibition of *M. domestica* ANAT

Inhibitor studies were carried out with an ANAT preparation (post-Hydopore AX) as described in the Experimental section.

Inhibitor	Acetyl CoA (20 μ M) ...	Activity remaining (% of control)	
		–	+
0.033 μ M PCMB		81	93
0.1 μ M PCMB		48	82
0.33 μ M PCMB		26	83
1.0 μ M PCMB		0	100
3.3 μ M PCMB		0	100
100 μ M Bromoacetanilide		N.D.	93
100 μ M Amethopterin		N.D.	76

Amino acid sequence

The N-terminal amino acid sequence of ANAT is given in Table 2. After the eleventh amino acid was successfully determined, the

signal degraded. Of the tryptic peptides produced, three were characterized but only peptides 2 and 3 gave uninterrupted sequences (Table 2).

Chemical inhibition studies

Incubating the enzyme with PCMB at pH 7.0 inhibited enzyme activity in a rapid and concentration-dependent manner (Table 3). Preincubation of the enzyme for 5 min with 20 μ M acetyl-CoA completely protected against inactivation by PCMB.

Incubating the enzyme with 100 μ M bromoacetanilide for 5 min had no effect on enzyme activity (Table 3). Similarly no inhibitory effect was observed with 100 μ M amethopterin. Both of these compounds have been shown to be potent inhibitors of the mammalian (Andres et al., 1988) and avian (Andres et al., 1983) ANAT enzymes.

DISCUSSION

The purification scheme described in this paper was found to be reliable and to give reproducible results. Although only small amounts (50–100 μ g) of homogeneous ANAT are obtained, the overall yield of enzyme activity from the original crude brei was reasonable at 17%. The purification procedure was simplified by the use of Blue Sepharose CL-6B at an early stage giving a purification of 200-fold in some instances. The group affinity ligand, Blue Dextran, has been used successfully in the separation of enzymes that require a nucleotide cofactor (see for example, Thompson et al., 1975).

The f.p.l.c. steps were very effective in achieving a rapid and significant purification of ANAT when working with relatively small amounts of protein. The widespread use of h.p.l.c. and f.p.l.c. techniques in protein purification is enabling, for the first time in many instances, proteins in low abundance to be isolated from insect sources (as described in Stapleton et al., 1989). The specific activity of *M. domestica* ANAT (3000 units/mg of protein) was considerably higher than that reported from rabbit liver (5.896 units/mg of protein; Andres et al., 1987), pigeon liver (14 units/mg of protein; Andres et al., 1983) and chicken liver (0.021 unit/mg of protein; Deguchi et al., 1988). This variation may represent varying degrees of enzyme purity, the method of protein determination, or enzyme inactivation.

As determined by several analytical methods, the ANAT purified by this protocol appeared to be homogeneous. The native M_r was 27 600 which was almost identical, within experimental error, to an M_r of 26 000 as determined for the denatured protein, clearly indicating that *M. domestica* ANAT is monomeric. The mammalian and avian enzymes are, by comparison, slightly larger with apparent M_r values of 33 000–34 000 (Andres et al., 1987; Deguchi, et al., 1988). The only M_r for insect ANAT is that reported by Maranda and Hodgetts (1977) for a crude preparation from *Drosophila melanogaster*, indicating an average value of 29 000 which is very similar to the value reported in this study.

The K_m value for tyramine of 9.0 μ M reported here is similar to that determined for *Drosophila* (6.3 μ M, Maranda and Hodgetts, 1977) and mosquito pupae (18.2 μ M, Shampengtong et al., 1987). The apparent pI value of 5.8 determined in this study is higher than the value of 4.8 reported for the avian liver enzyme (Andres et al., 1983).

It is interesting to note the duality of function exhibited by ANAT in both insects and mammals. In the latter case, the pineal 5-HT ANAT is thought to be distinct from the liver xenobiotic enzyme, whereas in insects it is not certain whether the ability to acetylate dopamine in the cuticle and biogenic amines in the nervous system resides in one or two separate

enzymes. Maranda and Hodgetts (1977) concluded from their study with *Drosophila* that the ability to acetylate dopamine and tyramine was consistent with a single molecular entity, although they could not unequivocally exclude the possibility of isoenzymes. In the present study the ANAT activity in adult flies, as determined using tyramine as substrate, is unlikely to be associated with the sclerotization of any cuticular structure. Haemocytes from newly eclosed (30 min) fleshfly, *Sarcophaga peregrina*, rapidly convert tyrosine, not into tyramine, but into dopa and then dopamine (Whitehead, 1970). This suggests that tyramine does not play an important role in providing precursors for cuticular sclerotization in Diptera. As previously noted, on two occasions a double peak of enzyme activity was observed during the chromatofocusing step; however, this observation was not followed up and further studies are essential before any clear conclusion can be drawn about the occurrence of isoenzymes. Therefore, although any cuticular dopamine *N*-acetyltransferase activity cannot be completely excluded, it is reasonable to suggest that the ANAT activity determined in this study is largely associated with the neuronal acetylation of biogenic amines, such as tyramine (Maxwell et al., 1980) and octopamine (Mir and Vaughan, 1981).

Detailed inhibitor studies of the mammalian liver ANAT have shown one reactive thiol group in the active site (Andres et al., 1988). Both iodoacetate and bromoacetanilide covalently react with a single cysteine residue, causing a complete loss of enzyme activity. A covalent acetyl-enzyme intermediate was isolated and identified as an acetylcysteinyl-enzyme species. Results obtained with the insect enzyme would suggest a similar active-site cysteinyl residue: 2-mercaptoethanol was essential for maintaining enzyme activity during the purification procedure and PCMB completely inhibited enzyme activity. This inhibitory effect could be reversed by preincubation with acetyl-CoA. In contrast, bromoacetanilide and amethopterin (Andres et al., 1983), both potent inhibitors of mammalian and avian ANAT, did not show any inhibitory action on the insect enzyme. This apparent difference in chemical sensitivity suggests that it may be possible to design specific inhibitors which have a high selectivity towards the insect enzyme.

The N-terminal and partial internal sequence of *M. domestica* ANAT represents the first amino-acid-sequence information for the insect enzyme. The N-terminal sequence does not show any similarity with data from the mammalian (Andres et al., 1987; Deguchi et al., 1988) and avian (Andres et al., 1983) enzymes. The amino acid sequence from chicken liver enabled the corresponding cDNA clone to be isolated (Ohsako et al., 1988). This work has led to the isolation of three cDNAs from human liver (Ohshko and Deguchi, 1990) that encode for different polymorphic and monomorphic isoenzymes of ANAT. It was suggested that the difference in activity between the two forms of

the polymorphic ANAT was due to a single amino acid substitution. Similar studies using oligonucleotide probes based on the *M. domestica* amino acid sequence and the mammalian ANAT cDNAs will facilitate the isolation of the corresponding insect ANAT cDNA clone(s). It is anticipated that such studies will provide important information on the distribution, regulation and presence of isoenzymes in different insect tissues and life stages.

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