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Catecholamine-β-alanyl ligase in the medfly *Ceratitis capitata*

M. Pérez, P. Wappner, L.A. Quesada-Allué *

Instituto de Investigaciones Bioquímicas, 'Fundación Campomar' and University of Buenos Aires, Patricias Argentinas 435, Buenos Aires 1405, Argentina

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Abstract

Dopamine (DA) and norepinephrine (NE) derivatives play an important role in the sclerotization and pigmentation of insect cuticles by serving as precursors for cuticular cross-linking. Protein preparations from prepupae of the medfly, *Ceratitis capitata*, were able to conjugate β-alanine with DA producing *N*-β-alanyldopamine (NBAD) or with NE, synthesizing *N*-β-alanylnorepinephrine (NBANE). The latter reaction has been demonstrated for the first time. Apparent kinetic parameters were obtained for both substrates, DA (V_{max}=30.7±6.0 pmol min^{-1} mg^{-1}; K_m=29.5±3.5 μM) and NE (V_{max}=16.1±6.6 pmol min^{-1} mg^{-1}; K_m=89.0±8.3 μM). The same protein seems to be responsible for both enzymatic activities, judging from several criteria like identical behavior under heat inactivation as well as identical Mg^{2+} and Mn^{2+} dependent stimulation and Co^{2+} inhibition. Furthermore, the melanic mutants *niger* of *C. capitata* and *ebony*^{4} of *D. melanogaster*, known to be defective for NBAD synthase, were also unable to synthesize NBANE. The protein preparation acylated tyrosine with much less efficiency, to produce sarcophagine (β-alanyltyrosine). Strikingly, extracts from the melanic mutants were unable to synthesize sarcophagine. Our results strongly suggest that the enzymatic activity previously known as NBAD synthase is in fact a novel catalytic protein showing broad substrate specificity. We propose to identify it as catecholamine-β-alanyl ligase.

Keywords: Dopamine; Norepinephrine; Cuticle; β-Alanine; Sclerotization; Sarcophagine; Medfly; *Ceratitis capitata*

1. Introduction

The main acquisition of arthropods through evolution is a shell providing ambient protection and acting as exoskeleton to support the body. Terrestrial insects and other arthropods acquired a waterproof hard cuticle, thus avoiding desiccation and gaining resistance to mechanical and chemical injury. To expand the body, the insect cuticle must be shed periodically, through a process called molting, triggered by 20-OH-ecdysone, a steroid hormone. The newly secreted cuticle is soft, pliant and colorless, and it becomes rigid and eventually colored as a consequence of sclerotization, a complex biochemical process that involves the cross-linking of proteins and chitin by reactive catecholamine-derived quinones. The presence of sclerotization precursors in the integument, namely *N*-β-alanyldopamine (NBAD), *N*-acetyldopamine (NADA) and *N*-β-alanylnorepinephrine (NBANE) has been described in a number of insects (Kramer and Hopkins, 1987; Wright, 1987; Sugumaran 1991, 1998; Hopkins and Kramer, 1992; Andersen et al., 1996). In general, NBAD is the main precursor of sclerotization in brown insect cuticles whereas NADA is used for making colorless or slightly colored cuticles (Hopkins and Kramer, 1992). Reactive derivatives (i.e. quinones and quinone methides) of both NBAD and NADA play a key role as final protein cross-linking agents (Sugumaran 1986, 1998; Andersen, 1991; Saul and Sugumaran 1988, 1990).

The in vivo synthesis of NBAD has been previously reported in different insects (Krueger et al., 1989; Morgan et al., 1987; Wappner et al., 1996a). In particular, we have been able to demonstrate the synthesis of [^{14}C]β-alanyldopamine following the injection of [^{14}C] β-alanine in prepupae (the 'zero time' of metamorphosis

Abbreviations: apf, after puparium formation; BAIA, β-amino isobutyric acid; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; GABA, γ-amino butyric acid; NADA, *N*-acetyldopamine; NBAD, *N*-β-alanyldopamine; NBANE, *N*-β-alanylnorepinephrine; NE, norepinephrine.

* Corresponding author. Tel.: +54-11-48634015; fax: +54-11-48652246.

E-mail address: lualque@iib.uba.ar (L.A. Quesada-Allué).

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as defined in Rabossi et al., 1991) of the medfly, *Ceratitis capitata*, just initiating sclerotization and pigmentation of the larval cuticle (Wappner et al., 1996a,b). The tanned puparium formed by these processes is reddish-brown and will enclose the metamorphosing insect until eclosion of the adult.

It has been previously shown that the *Drosophila melanogaster ebony* mutant (Jacobs and Brubaker, 1963; Hodgetts and Knopka, 1973) and the melanic mutant *niger* of *Ceratitis capitata* (Wappner et al., 1996a,b; Pérez et al., 1997) lack NBAD because they cannot conjugate β-alanine with dopamine (DA). This was demonstrated in experiments in which pupariating *niger* medflies were unable to use injected β-alanine for tanning the puparium (Wappner et al., 1996b). In this mutant, as in other similar melanic insects like the melanic swallowtail butterfly (Koch et al., 2000), non-conjugated DA enters the melanin pathway, thus producing an excess of it and generating a shiny black cuticle instead of the typical brown one. We also demonstrated that most of the NBAD synthesis occurs in the epidermis since variegated insects were obtained when heterozygous embryos were irradiated (Wappner et al., 1996a).

The cell-free synthesis of NBAD in *Drosophila melanogaster* was mentioned in a review by Wright (1987), but this finding has not yet been formally published. We previously reported the cell-free synthesis of [^{14}C]β-alanyldopamine by crude extracts of *Ceratitis capitata* and those of other insects including *Drosophila melanogaster* (Wappner et al., 1996a; Pérez et al., 1997). We have also been able to confirm that *C. capitata niger* and *D. melanogaster ebony* do not have a functional NBAD synthase (Pérez et al., 1997). It has been recently shown that this enzyme plays a similar role in the melanic mimicry of females of the North American swallowtail butterfly *Papilio glaucus* (Koch et al., 2000).

Sugumaran, 1991 and Saul and Sugumaran, 1990, demonstrated that NBANE is formed by a non-enzymatic reaction of NBAD quinone methide with water. It is also assumed that NBANE is synthesized in the integument through β-hydroxylation of NBAD (Hopkins and Kramer, 1992). As far as what is currently known, no other alternative pathway of NBANE synthesis has been reported.

Here we present results showing that medfly prepupae protein preparations support the synthesis not only of NBAD but also of NBANE, and that a single protein seems to be responsible for both enzymatic activities.

2. Materials and methods

2.1. Insects

Wild type *C. capitata* (INTA Arg 17) strain and the mutant strain *niger* (*nig*^{1} allele) were reared in carrot-based medium as described by Quesada et al. (1994). Larvae and adult flies were maintained at 23°C, 60–80% relative humidity and a 16L:8D photoperiod. All of the results below refer to time-dependent events occurring during the standard life cycle under these controlled culture conditions (Rabossi et al., 1992). The insect cultures were synchronized and the exact age assessed using a binocular microscope. The fly age within the puparium is expressed in hours after puparium formation (apf), starting from the definitive immobilization of the third-instar larva defined as zero time (Rabossi et al., 1991). Wild type *D. melanogaster Oregon R* and the mutant strain *ebony*^{4} were reared in commercial fly medium and maintained at 18°C.

2.2. Chemicals

Dopamine (DA), *N*-acetyldopamine (NADA), 3,4-dihydroxyphenylalanine (DOPA), norepinephrine (NE) and β-alanine were from Sigma Chem. Co. [^{14}C]β-alanine was from New England Nuclear (54.5 μCi μmol^{-1}) and from American Radiolabeled Chemicals (55 μCi μmol^{-1}). *N*-β-Alanyldopamine (NBAD) and *N*-β-alanyldopa were synthesized as described by Yamasaki et al. (1990) with slight modifications. *N*-β-Alanylnorepinephrine (NBANE) was a generous gift from Dr K. Kramer (USDA-ARS, Manhattan, KS, USA). All the solvents used were of the highest purity available.

2.3. Biosynthesis of β-alanine derivatives

To measure the cell-free synthase activities, 5 g of 4 h apf prepupae were homogenized in 10 ml cold Buffer 'A': 50 mM sodium borate buffer, pH 8.2, saturated with phenylthiourea (PTU) and containing 10 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 2 mM DTT, 10% glycerol and a mixture of protease inhibitors (E-64, leupeptin, pepstatin and PMSF) as described (Wappner et al., 1996a). After centrifugation at 12,000 g (4°C, 10 min), the soluble material in the supernatant was subjected to salting out with ammonium sulfate and the 30–70% precipitate was dissolved in 2.5 ml of Buffer A and dialyzed during 1 h 40 min at 4°C against 50 mM sodium borate buffer, pH 8.2. This slightly purified dialyzed protein extract was immediately used as source of enzymes or frozen in aliquots at −70°C. The standard reaction mixture contained 50 mM sodium borate buffer, pH 8.2, 2 mM ATP, 5 mM $MgCl_2$, 0.1 mM substrate (DA, DOPA or NE), 5×10^5 counts/min (cpm) of [^{14}C]β-alanine and 3 μl of enzymatic extract in a final volume of 50 μl. (The latter additionally contributed to a final 2.4 mM 2-mercaptoethanol, 0.12 mM DTT and 0.6% glycerol.) The samples were incubated for 15 min at 22°C, and the reaction was stopped by the addition of perchloric acid to final concentration of 150 mM. We knew from previous results (Wappner et al., 1996a) that

the extracts also contain a NBAD hydrolase which is not active in our standard conditions for synthesis. In preincubation experiments (Fig. 4), the enzyme extract (3 μl) was preincubated in the same mixture as above without catecholamines, ATP or [^{14}C]β-alanine, which were added last to start the reaction. To synthesize sarcophagine, tyrosine was used as a substrate instead of catecholamines.

2.4. Catecholamine and β-alanine analysis

Incubation products were isolated using alumina columns as described by Wappner et al. (1996a) and analyzed by reversed phase HPLC (Econosphere-C-18, Altech) exactly as described earlier (Wappner et al., 1996a,b). TLC was carried out using methyl-ethylketone–propionic acid–water (40:13:11). Radioactivity in the samples was measured in a Rackbeta 1214 Pharmacia liquid scintillation counter using Ultima-gold (Packard) as the scintillator fluid. Protein concentration was determined using the Folin reagent and BSA as a standard.

3. Results

3.1. Cell-free synthesis of NBAD

Extracts from the medfly *C. capitata* in the early prepupal stage (Rabossi et al., 1992) (exactly timed at 4 h apf) were fractionated by salting out with 30–70% ammonium sulfate and, after dialysis, immediately used as a source of enzyme. At this early stage of metamorphosis, the synthesis of NBAD was found to be associated with the integument, i.e. the epidermis together with the old larval cuticle undergoing a transformation to give rise to a puparium. When the prepupae were dissected to prepare separate homogenates of tissues, only traces of enzymatic activity could be detected in the internal organs, most of them initiating histolysis, whereas about 94% of the whole insect enzymatic activity remained with the integumental carcass. Strikingly, using these cell-free synthesis conditions, extracts of ganglia and brain also were able to synthesize NBAD but only at trace levels relative to the integument. In terms of arbitrary units of enzymatic activity the dissected brain and ganglia contained no more than 1.8% of the activity measured in the whole body.

To isolate the newly synthesized NBAD, we have taken advantage of the ability of alumina columns to absorb diphenols, in particular catecholamines and its derivatives (Wappner et al., 1996a; Anton and Sayre, 1962). Table 1 and Fig. 1(A) show that the medfly protein preparation incubated with DA and β[^{14}C]-alanine was able to synthesize a single substance, eluted from the alumina column and identified by HPLC as

Table 1
Biosynthesis of [^{14}C]β-alanine conjugates by protein preparations from *C. capitata* (The enzymatic proteins were incubated under standard conditions (see Section 2) with 0.1 mM one or two catecholamines and the labeled reaction products isolated using alumina columns and counted. Experiments were conducted in triplicate and repeated with more than 10 different enzymatic preparations. See Fig. 1 for HPLC identification of the synthesized substances.)

Substrate	cpm	pmol min^{-1} mg^{-1}	Activity (%)
DA	17,211	147±10.50	100.0
DA+NE	17,186	146±10.44	99.8
DA+DOPA	16,047	137±5.20	93.2
NE	16,734	143±7.15	97.2
DOPA	7624	65±5.56	44.0
DA+GABA[a]	15,369	131±6.35	89.3
DA+BAIA[a]	15,506	132±3.00	90.1

[a] 1 mM GABA and BAIA.

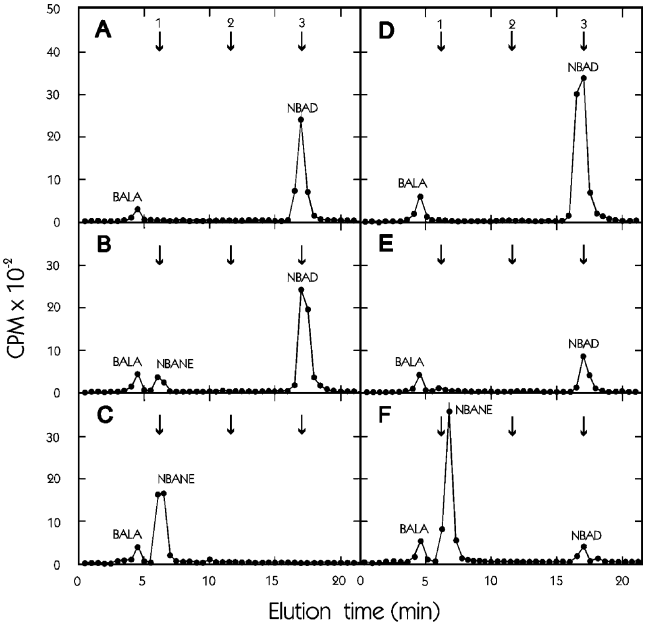

Fig. 1. HPLC analysis of β-alanyl-catechol conjugates from the incubation of potential precursors with protein preparations from *C. capitata*. The [^{14}C]-labeled products from incubations described in Table 1 were analyzed by reversed phase HPLC together with standards. The incubation mixtures contained [^{14}C] ß-alanine (BALA) and (A) DA, (B) DA+NE, (C) NE, (D) DA+DOPA, (E) DOPA, (F) NE+DOPA. The arrows indicate the retention time of chemically synthesized standards (1) NBANE, (2) *N*-ß-alanyldopa and (3) NBAD, detected as UV absorbing at λ_{280}.

β[^{14}C]alanyldopamine, at an apparent V_{max} of 30.7±6.0 pmol min^{-1} mg^{-1} protein (Fig. 2(A)). As shown in the inset to Fig. 2(A), in our conditions, the apparent K_m for DA was 29.5±3.5 μM. The synthesis of NBAD required Mg^{2+} or Mn^{2+} cations whereas 5 mM Co^{2+} inhibited the reaction totally (Fig. 3). The addition of 5 mM Cu^{2+} to the standard mixture containing 5 mM Mg^{2+} also inhibited the reaction completely. Apparently,

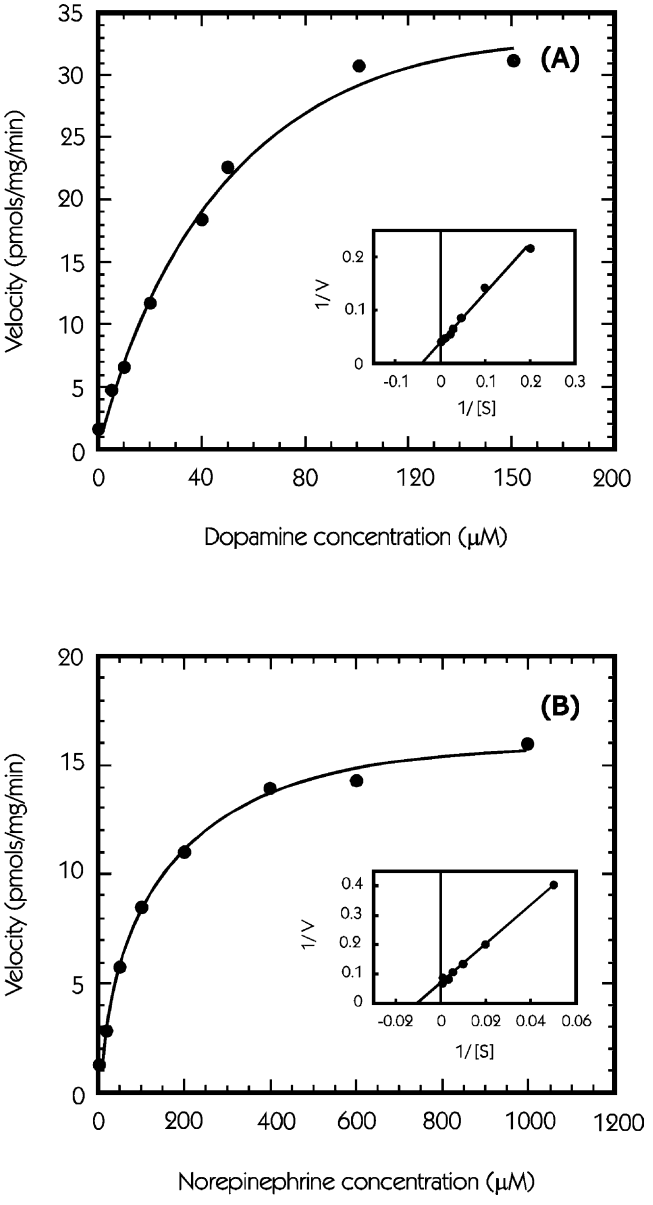

Fig. 2. Effect of varying concentration of DA or NE on synthase activity in protein preparations from *C. capitata* wild type prepupae. (A) Kinetic constants estimated using DA as substrate. Assay was performed under standard conditions except that the concentration of DA was varied from 5 to 150 μM. Inset: double reciprocal plot; abscissa=1/*S* (μM), ordinate=1/*V* (pmol min^{-1} mg^{-1}). Apparent kinetic constants were: V_{max} 30.7±6.0 pmol min^{-1} mg^{-1}; K_M 29.5±3.5 μM (*n*=3, mean±SEM). (B) Kinetic constants estimated using NE as substrate. Assay was performed under standard conditions except that the concentration of NE was varied from 20 to 1000 μM. Inset: double reciprocal plot; abscissa=1/*S* (μM), ordinate=1/*V* (pmol min^{-1} mg^{-1}). Apparent kinetic constants were: V_{max} 16.1±6.6 pmol min^{-1} mg^{-1}; K_M 89.0±8.3 μM (*n*=3, mean±SEM).

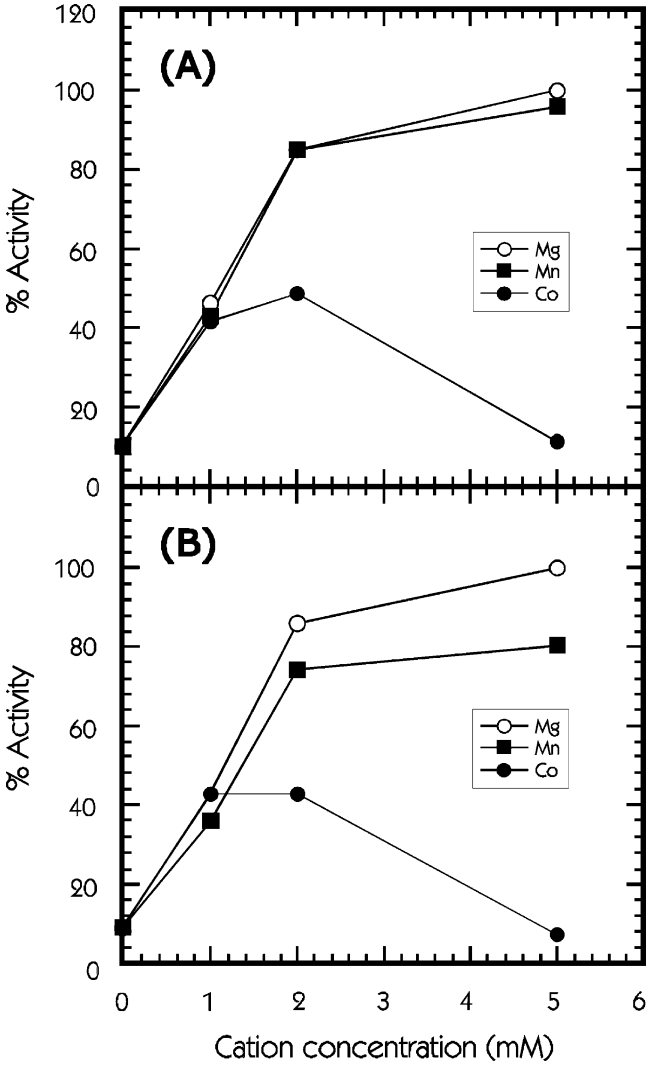

Fig. 3. Effect of divalent cations on the biosynthesis of NBAD and NBANE by protein preparations from *C. capitata*. $MgCl_2$, $MnCl_2$ or $CoCl_2$ were tested at different concentrations on the biosynthesis of (A) NBAD and (B) NBANE. Results are expressed as percent of the standard assay containing 5 mM $MgCl_2$.

no Coenzyme A, Acetyl-CoA or pyridoxal phosphate are required in the reaction since they were unable to increase the enzymatic activity (not shown). In spite of the relatively short time of dialysis, it is unlikely that traces of another small MW co-factor might be involved in the reaction. We know that after heating the enzymatic preparation at 90°C and discarding the denatured protein, when the supernatant was added to the incubation mixture it was unable to enhance the reaction, thus showing that no heat-resistant stimulating factor was originally present in the extract (not shown).

The enzymatic activity conjugating β-[^{14}C]alanine and DA was found to be quite stable when stored at −65 to −70°C (half-life approximately one year), but the enzymatic protein, even in crude extracts without dialysis, was very unstable at higher temperatures, thus making the enzyme purification difficult that has not been achieved yet. The half-life of the enzyme when preincubated at −20°C in the presence of buffer and 5 mM $MgCl_2$ was 16 h and only about 8 h when preincubated at 4°C (not shown). Fig. 4 shows the time-dependent decay of the enzymatic activity when preincubated at 22°C, the

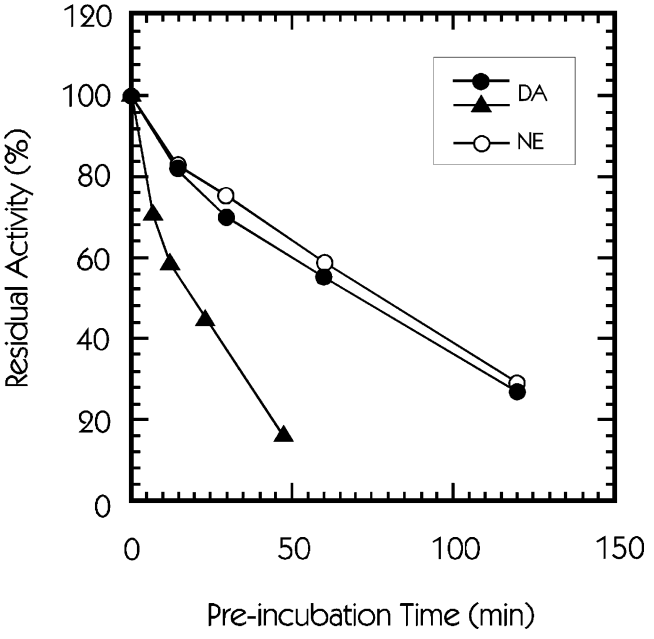

Fig. 4. Time-dependent decay of the enzymatic activity in protein preparations from *C. capitata*. The extracts were preincubated at 22°C (●) or 25°C (▲) for the indicated times and then incubated as in Table 1 with DA to obtain NBAD. Similar experiments were done at 22°C and then incubated under standard conditions with NE to obtain NBANE (○). Synthesis is expressed as percent of the non preincubated control.

half-life being 70 min. At 25°C the half-life was only 12 min (Fig. 4). Therefore, the extracts had to be tested shortly after homogenization and, in general, no more than two purification steps could be implemented before the loss of activity. Many of the known chemicals used for enzyme stabilization have been tested for storing the extracts, with little success, the most useful being 20% glycerol. The enzymatic preparation contains a cocktail of protease inhibitors (see Section 2). Moreover when preincubations as in Fig. 4 were carried out in the presence of additional E-64, Pepstatin A and PMSF, no changes in NBAD-synthase stability occurred, thus showing that the loss of activity is not due to an active protease present in the extract.

3.2. Cell-free synthesis of NBANE

To look for competitive inhibitors of the enzyme, NE or 3,4-dihydroxyphenylalanine (DOPA) were included in the incubation mixture. As shown in Table 1, none of these substances significantly decreased the amount of labeled material that was synthesized by the extract. β-Aminoisobutyric acid (BAIA) and γ-aminobutyric acid (GABA) do not inhibit the reaction (Table 1) and we know that 10-fold L-alanine was unable to diminish the amount of the synthesized NBAD (Wappner et al., 1996a). Surprisingly, the HPLC analysis of the alumina-bound radiolabeled material obtained when NE was incubated together with DA revealed the presence of a substance behaving in HPLC exactly like the NBANE standard, in addition to NBAD (Fig. 1(B)). Furthermore, when NE was used as the sole catecholamine substrate instead of dopamine, NBANE was synthesized as an exclusive reaction product (Table 1, Fig. 1(C)) at an apparent V_{max} 16.1±6.6 pmol min^{-1} mg^{-1} protein (Fig. 2(B)). The apparent K_m was 89.0±8.3 μM (inset to Fig. 2(B)) whereas the pH-dependent behavior (not shown), divalent cation specificity (Fig. 3) and heat sensitivity (Fig. 4) were similar to those required for NBAD synthesis. The identity of the labeled NBANE was confirmed by analytical TLC and acid hydrolysis assays (not shown). Treatment of the synthesized NBANE with 1.2 N HCl for 2 or 4 h at 95°C released β-[^{14}C]alanine, consistent with the expected conditions necessary to cleave a β-alanine–catechol linkage (Hopkins et al., 1984). This is the first time such cell-free biosynthesis using NE as substrate has been demonstrated in any organism.

Morgan et al. (1987) postulated that when NBAD was incubated with epidermal extracts of *M. sexta* NBANE was formed via ß-hydroxylation (Morgan et al., 1987). Working with *Sarcophaga bullata*, Saul and Sugumaran, 1990, demonstrated that NBANE arises by a non-enzymatic reaction of water with NBAD quinone methide. The latter is formed from NBAD by way of NBAD quinone, by the action of phenoloxidase and quinone isomerase.

No NBANE formation was detected in experiments like those in Fig.1(A). Moreover, when [^{14}C]NBAD was incubated with similar extracts no conversion to NBANE could be detected and all the original [^{14}C]NBAD was recovered, even after 2 h of incubation. However, we cannot exclude the possibility of direct conversion of NBAD to NBANE by *C. capitata* extracts, because 2.4 mM 2-mercaptoethanol is present in the incubation mixture and therefore the possibility exists of NBAD quinone reduction back to NBAD (Sugumaran, 1991).

3.3. Presence of DOPA-decarboxylase in the extract

The fact that NE turned out to be a substrate for NBANE synthesis led us to investigate whether L-DOPA is also used as substrate to synthesize the corresponding derivative. As shown in Table 1, incubation with both DA and DOPA showed no apparent inhibition of the product synthesis and HPLC analysis (Fig. 1(D)) revealed a profile of radioactivity identical to that in Fig.1(A), with NBAD as the only labeled reaction product (see subsequently). Furthermore, incubation of [^{14}C]ß-alanine together with L-DOPA as the only unlabeled substrate (Table 1) led to accumulation of a labeled product that showed a retention time in the HPLC column coincident with that of NBAD and different from that of the synthetic *N*-ß-alanyldopa (Fig. 1(E)). This

chromatographic behavior was maintained in different mobile phases. The labeled product also co-migrated with a NBAD standard in TLC (data not shown). The possibility that an unknown putative DOPA derivative other than *N*-ß-alanyldopa, might be formed and behaved exactly like NBAD in our HPLC and TLC systems, in different solvents, appears highly unlikely. The most logical explanation appears to be a rapid decarboxylation of L-DOPA to give rise to DA, thus accounting for these results. Indeed, when only L-DOPA was incubated with [^{14}C]ß-alanine as in Fig. 1(E), an unlabeled substance was formed, showing in our HPLC system identical retention time to that of DA. Moreover, when L-DOPA was incubated alone with the extract under conditions that were appropriate for DOPA-decarboxylase activity (Black and Smarrelli, 1986), 2/3 of L-DOPA was transformed in DA in 10 min (data not shown). When L-DOPA was incubated together with NE, the synthesis of normal amounts of NBANE occurred whereas only a small amount of NBAD was synthesized (Fig. 1(F)). Thus, to obtain NBAD from DOPA, DA must be generated first by DOPA-decarboxylase present in the extract and when NBAD formation does occur (Fig. 1(E)), DA is the limiting precursor.

3.4. Cell-free synthesis of sarcophagine (β-alanyltyrosine)

The only reported enzymes having catalytic characteristics related to those of the one described here are the insect fat body sarcophagine synthase, conjugating ß-alanine with tyrosine (Dunn et al., 1977; Kano and Natori, 1984) and the vertebrate carnosine synthase, which conjugates ß-alanine with histidine (Wood and Johnson, 1981). We tested our *C. capitata* extracts with the objective of synthesizing carnosine (β-alanylhistidine) and, to date, only negative results were obtained under several conditions. The conjugation of tyrosine and [^{14}C]ß-alanine was also tested using the same conditions required to synthesize NBAD and a labeled substance behaving, during HPLC chromatography, like sarcophagine was obtained (Fig. 5(A)). In the absence of exogenously added tyrosine, a small amount of β-alanyltyrosine can be detected (Fig.5(A)), thus reflecting the amount of endogenous tyrosine.

3.5. Synthesis of NBANE and sarcophagine in melanic mutants

Extracts of the *C. capitata* mutant *niger* and of *D. melanogaster* mutant *ebony*, which are known to be unable to synthesize NBAD (Pérez et al., 1997; Wappner et al., 1996a,b; Jacobs and Brubaker, 1963; Hodgetts and Knopka, 1973), were tested for their ability to conjugate NE and β-alanine. Table 2 shows that none of the mutant extracts were able to synthesize NBANE. This is a very strong indication that a single enzyme exhibits both NBAD and NBANE synthase activities. Strikingly, no sarcophagine was synthesized in the melanic mutant *niger* (Fig. 5(B)), thus reinforcing the idea of a single locus involved in the biosynthesis of NBAD, NBANE and ß-alanyltyrosine.

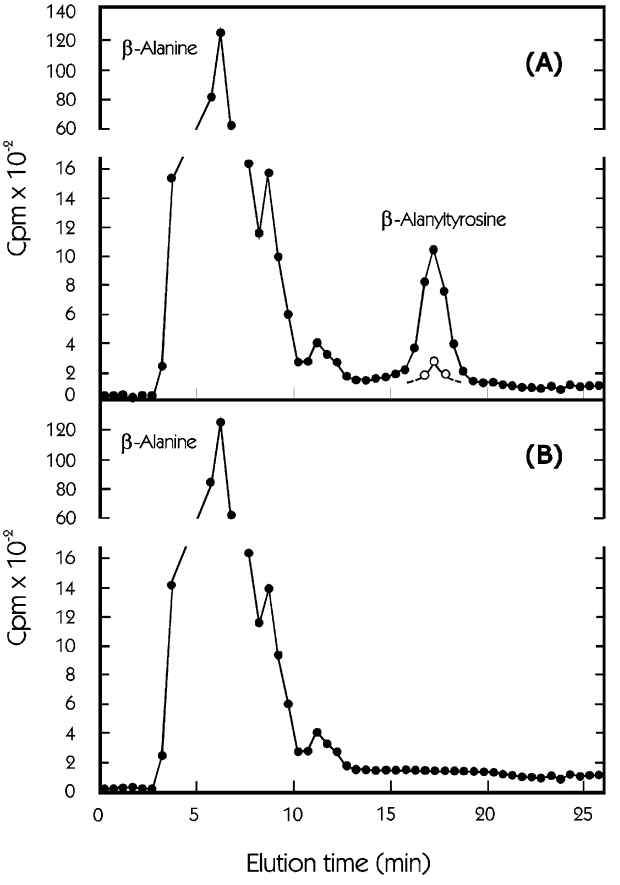

Fig. 5. Synthesis of sarcophagine by protein preparations from *C. capitata*. The [^{14}C]-labeled products from incubations of wt extracts (A) or *niger* extracts (B) as described in Section 2 were analyzed by reversed phase HPLC. The incubation mixtures contained [^{14}C]ß-alanine and tyrosine (●), or [^{14}C]ß-alanine alone, without tyrosine (○).

Table 2
Biosynthesis of NBAD and NBANE by extracts from wt and melanic mutants of *C. capitata* and *D. melanogaster* (Extracts from wt and melanic insects were incubated with 0.1 mM DA or NE as in Table 1, to test the ability to synthesize NBAD or NBANE. The data are the mean of triplicate experiments.)

Insect	NBAD (pmol min^{-1} mg^{-1})	NBANE (pmol min^{-1} mg^{-1})
C. capitata		
wt	147.00±10.5	146.00±10.44
niger	0.10±0.01	0.06±0.005
D. melanogaster		
wt	3.26±0.28	2.48±0.22
ebony	0.06±0.001	0.04±0.001

4. Discussion

From the above results, we can postulate that the medfly enzymatic activity synthesizing NBAD seems to be a broad-specificity ligase (see subsequently), capable of using alternatively DA or NE as substrate. The results in Fig. 4 on extracts undergoing decay in the enzymatic activity synthesizing NBAD and NBANE, under heat treatment, support this notion, since the temporal decrease in product formation was identical with both DA and NE substrates. The optimum pH 8.2 and the pH-dependent profile were the same (not shown). The assays using different concentrations of divalent cations pointed to the same conclusion. Fig. 3 shows identical stimulation by the co-factors Mg^{2+} and Mn^{2+} and the same degree of inhibition by Co^{2+}. Five milimolar Cl_2Zn or Cl_2Cd inhibited 97% of both the reactions. Finally, the strong results on the absence of synthesis by melanic mutants reinforce the hypothesis that the same enzyme is expressed from *ebony* or *niger* genes and is responsible for the synthesis of NBAD and NBANE. Furthermore, the absence of synthesis of sarcophagine in these mutants indicates an even broader specificity for the proposed novel enzyme. It is unlikely but still possible that different protein products might exist, due to an alternative transcript splicing. Another possibility would be that the product of the *niger* (or *ebony*) gene might be the enzyme subunit that contains the basic catalytic domain, whereas other putative subunits might influence substrate specificity. However, in *D. melanogaster*, no mutants of other loci showing the *ebony* biochemical phenotype have been ever described.

A similar broad substrate specificity has been reported for other enzymes of the catecholamine pathway like *N*-catecholamine-acetyl-transferase or DOPA-decarboxylase (Brown and Nestler, 1985; Dewhurst et al., 1972). Apparently no co-factors like Coenzyme A, Acetyl-CoA or Pyridoxal phosphate are involved in the reaction. Everything seems to indicate that ATP provides the energy for the direct conjugation of ß-alanine with a catecholamine (or tyrosine), without the formation of an intermediate. Therefore, the enzymatic activity described here should be considered a catalytic protein with an activity of catecholamine-β-alanine ligase of broad specificity rather than a NBAD transferase, as previously considered (Wappner et al., 1996a). As discussed above, we cannot discard that the other unknown proteins might confer substrate specificity. The other diphenols and monophenols will be tested as substrates in the future to further define the specificity of this important insect enzyme. Preliminary results indicate that 3-4-dihydroxyphenyl-acetic acid is a poor substrate.

We have demonstrated that the enzymatic activity described here requires the respective expression of *C. capitata niger* and *D. melanogaster ebony* genes. Significantly, a recent report (Hovemann et al., 1998) showing the sequence of the *D. melanogaster ebony* gene, postulates that the predicted protein product is related to a family of peptide synthases.

Our results show, for the first time, the existence of a novel type of enzyme catalyzing, in addition to NBAD formation, the previously unknown β-alanylation of NE. The resulting substance, NBANE, which is the major extractable catecholamine in cockroaches (Czapla et al., 1990), is known to be present in cuticles of other insect species. For example, it is necessary for normal tanning of *Manduca sexta* pupal cuticle (Roseland et al., 1986; Morgan et al., 1987). Therefore, we assume that the biosynthesis of NBANE reported here represents a physiological synthesis and that, depending on the substrate availability in the epidermis and other tissues and on the affinity, NBAD, NBANE or a mixture of both will be synthesized. The enzyme showed more affinity for DA than for NE, and in our cell-free conditions the respective ß-alanine conjugates were obtained simultaneously (in a 8:1 proportion) when both substrates were present, (Fig. 1(B)).

No consumption of NBAD or conversion to NBANE occurred in our experimental conditions (see Section 3). However, Sugumaran (1987, 1988) and Saul and Sugumaran (1990), showed that NBANE arises from NBAD through the sequential action of phenoloxidase generating NBAD quinone and quinone isomerase generating NBAD quinone methide. The latter in turn forms NBANE by non-enzymatic reaction with water. As discussed above, in our assays we were unable to detect this reaction, probably due to the presence of small amounts of 2-mercaptoethanol and traces of DTT. It has also been reported that NBAD is a direct precursor of NBANE by way of β-hydroxylation (Morgan et al., 1987; Czapla et al., 1990). Thus, it seems that there is more than one biosynthetic pathway in the formation of NBANE.

As shown, when DOPA was incubated with the protein preparation, no ß-alanyldopa was synthesized, whereas DA was formed and used as substrate to generate NBAD. These data are consistent with the fact that *N*-ß-alanyldopa has not been detected in any cuticular system. *N*-Acetyldopa was found to be present in the ganglia of the locust *Schistocerca gregaria* (Vaughan and Neuhoff, 1976) and β-alanyldopa has been reported as precursor and part of the antibacterial substance *N*-β-alanyl-5-*S*-glutathionyl-3-4-dihydroxyphenylalanine (5-*S*-GAD) in the flesh fly *Sarcophaga peregrina* (Leem et al., 1996).

The biosynthesis, mediated by the medfly crude extracts, of β-alanyltyrosine (sarcophagine), previously reported only in *Sarcophaga* (Dunn et al., 1977; Kano and Natori, 1984) was actually expected due to the evolutionary relatedness of both cyclorrhaphous dipterans. However, the absence of sarcophagine synthesis in the

melanic mutants was a surprising result, pointing to a very broad specificity of the same ligating enzyme.

Since the substrates of the above described catecholamine-β-alanyl ligase, DA and NE are well-known neurotransmitters, the activity of this ligase in the nervous system remains to be tested. Preliminary experiments using dissected brain and ganglia as a cell-free enzyme source showed that small amounts of a substance behaving like NBAD were synthesized by the extracts. It is important to note that the melanic mutants *D. melanogaster ebony* (Hotta and Benzer, 1969) and *C. capitata niger* (unpublished results), in addition to the black cuticle phenotype, display behavioral disorders. Therefore, if confirmed, the putative synthesis in the nervous tissue of NBAD and NBANE may suggest a role for the enzyme described here in the metabolism of neurotransmitters.

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