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Characterization of a new enzyme system that desaturates the side chain of *N*-acetyldopamine

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A novel enzyme system that desaturates the side chain of the catecholamine derivative, *N*-acetyldopamine (NADA), was isolated and characterized from the larval cuticle of *Sarcophaga bullata*. The NADA desaturase system which converts NADA to 1,2-dehydro-NADA, surprisingly, does not resemble dehydrogenases such as succinate dehydrogenase. It uniquely performs the desaturation reaction by oxidizing NADA to its corresponding quinone and subsequently converting the resultant quinone to 1,2-dehydro-NADA via NADA quinone methide. Accordingly, desaturase enzyme preparation contained both *o*-diphenoloxidase activity and NADA quinone:NADA quinone methide isomerase activity. In addition, inhibition studies as well as trapping experiments also confirmed the obligatory formation of NADA quinone as the transient intermediate of the NADA desaturation. It is the first report of a cell-free system causing the side chain desaturation of any catecholamine derivative.

Catecholamine metabolism; Dehydro-*N*-acetyldopamine; Cuticular tanning; Quinone methide; Quinone isomerase; Phenoloxidase; (*Sarcophaga bullata*)

1. INTRODUCTION

The key roles of dopa and dopamine in the biosynthesis of (i) the neurotransmitter noradrenaline, (ii) the hormone adrenaline, (iii) the sclerotizing precursors, *N*-acyldopamines, for cuticular tanning in insects and (iv) the pigments eumelanin and pheomelanin, are well-established [1–3]. In order to serve in such diverse biological processes, these two compounds undergo a variety of enzymatic transformations including oxidation to quinone, side chain hydroxylation, methylation, amine oxidation, acylation and conjugation [1–3]. Although side chain desaturation is a possible reaction and has been invoked as a key reaction for tanning of insect cuticle [3–5], the enzyme system responsible for the introduction of the double bond on the catecholamine side chain has never been isolated or characterized. In this communication, we report for the first time, the isolation and characterization of a new enzyme system which desaturates the catecholamine derivative, NADA. Interestingly, unlike other known dehydrogenases, the NADA desaturase system accomplishes its reaction by initially oxidizing NADA to its quinone and subsequently isomerizing the NADA quinone to dehydro-NADA via NADA quinone methide.

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Abbreviations: dehydro-NADA, 1,2-dehydro-*N*-acetyldopamine; NADA, *N*-acetyldopamine; NANE, *N*-acetylnorepinephrine

2. MATERIALS AND METHODS

2.1. *Partial purification of NADA-desaturase system*

All operations were carried out at 0–5°C unless stated otherwise. Larvae of *Sarcophaga bullata* were raised on a dog food diet at 37°C. At the wandering stages, the larvae (275 g) were collected, washed extensively with distilled water and homogenized in ice-cold water with a Virtis Omnimixer at maximum speed for 30 s. Cuticle sheaths were recovered by sieving the homogenate through a 100 μm screen. After extensively washing the cuticle with water, it was rehomogenized for an ad-

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ditional 2 min with ice-cold water and filtered. The recovered cuticle was suspended in 200 ml of 0.1 M sodium borate buffer, pH 8.5, for 2 h. Following this treatment, the mixture was passed through cheese cloth. The filtrate was treated with solid ammonium sulfate and brought to 30% saturation. After centrifugation at 10000 × *g* for 10 min, the sediment was discarded. The clear supernatant was brought up to 60% saturation with respect to ammonium sulfate and centrifuged at 10000 × *g* for 10 min. The precipitate was collected, dissolved in 5% ammonium sulfate and loaded onto a column (2.5 × 5 cm) of phenyl-Sepharose equilibrated with 5% ammonium sulfate. After loading the sample, the column was washed extensively with the same solution (approx. 100 ml). Elution of bound proteins was achieved by 0.025 M Tris-HCl buffer, pH 7.5. The eluate was used as the partially purified desaturase system.

2.2. *HPLC analysis*

HPLC analysis of reaction mixture was carried out using a Beckman (Berkeley, CA) model 332 liquid chromatography system equipped with two model 110 B pumps, a model 420 controller, a model 160 absorbance detector and a model 427 integrator. Separations were achieved on a Beckman C_{18}-IP ultrasphere reverse-phase column (5 μm, 4.6 × 250 mm) with the isocratic solvent system – 0.05 M acetic acid containing 0.2 mM sodium octylsulfonate in 30% methanol at a flow rate of 0.6 ml/min (System I).

Alternatively, a second HPLC system (System II) equipped with two model 100 A Altex pumps, a model 420 controller, a Hitachi model 110-10 spectrophotometer, and an Altex model C-RIA integrator was also used. Separations were achieved in this system on a Beckman C_{18}-ultrasphere reverse-phase column (5 μm, 4.6 × 50 mm) with a starting buffer of 0.05 M acetic acid containing 0.2 mM sodium octylsulfonate in 30% methanol at a flow rate of 1.0 ml/min. At 5 min, the methanol composition was changed to 40% and the flow rate increased to 1.5 ml/min until the end of the run (usually 20 min).

2.3. *Other procedures*

Ultraviolet and visible spectral studies were carried out using a Gilford model 2600 spectrophotometer. The authentic *N*-acetylcysteinyl-NADA quinone adduct was a generous gift of Dr Hemalata Dali. NADA and *N*-acetylcysteine were obtained from Sigma Chemical Co., St. Louis, MO; NANE was prepared by acetylation of norepinephrine [6]. Dehydro-NADA was synthesized by one of two published procedures [6,7].

3. RESULTS AND DISCUSSION

Fig.1 gives the ultraviolet spectral studies accompanying the oxidation of NADA by the desaturase system. The appearance of a shoulder in the UV spectrum of the reaction mixture at about 320 nm was consistent with the formation of a product which has a conjugated double bond (or a carbonyl group) to the catecholic nucleus. In order to identify the product(s) formed in the reaction, HPLC studies were carried out.

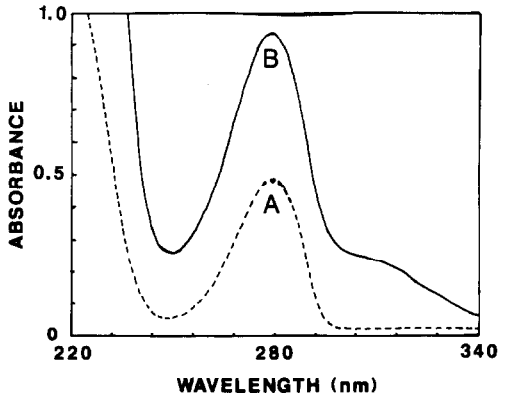

Fig.1. Ultraviolet spectral changes accompanying the oxidation of NADA by NADA-desaturase system. A reaction mixture containing 2 mM NADA, 600 μg of desaturase preparation in 50 mM sodium phosphate buffer, pH 5.5, was incubated at room temperature for 2 min and the ultraviolet spectral changes accompanying the enzymic oxidation were recorded. (A) Control reaction without the enzyme, (B) complete reaction mixture.

Fig.2 shows the HPLC analysis of the NADA-desaturase reaction. Apart from unreacted NADA (2.79 min peak), the presence of two new compounds in the reaction mixture can be witnessed. The compound eluting at 1.85 min was identified to be NANE, based on HPLC retention times, cochromatography and ultraviolet spectroscopy. Earlier, we have demonstrated that NADA conversion to NANE is caused by the combined action of phenoloxidase and quinone isomerase and nonenzymatic hydration of resultant NADA-quinone methide [8–10]. Accordingly, the desaturase preparations contained both of these enzyme activities.

The second compound eluting at 3.88 min (fig.2) corresponded to dehydro-NADA. Thus, both this material and dehydro-NADA exhibited the same retention times under different HPLC conditions in different HPLC systems, and comigrated as a single symmetrical peak in various solvents. Upon enzymatic oxidation by mushroom tyrosinase, it readily generated dehydro-NADA dimers as the synthetic compound [11]. Finally, the UV spectrum of the product (fig.3) was indistinguishable from that of synthetic dehydro-NADA, thereby confirming the identity.

Dehydro-NADA could be formed either directly from NADA by a specific desaturase, as suggested by Andersen [4,5], or indirectly from NADA

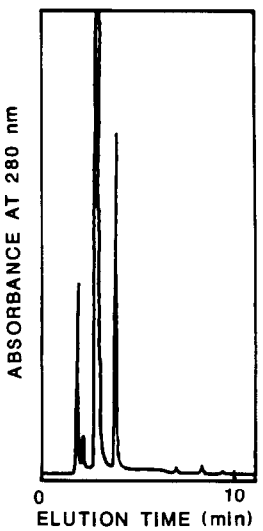

Fig.2. HPLC analysis of NADA-desaturase reaction mixture. The reaction mixture outlined in the legend to fig.1 was subjected to HPLC analysis using conditions described in section 2.2 with HPLC system I. The peaks at 1.85, 2.79 and 3.88 are due to NANE, unused NADA and dehydro-NADA, respectively. The minor peak at 2.17 min is due to NADA quinone.

quinone methide by tautomerization [3,12,13]. Although conversion of NANE formed in the reaction mixture to the dehydro-NADA by a specific dehydrase is an alternative route, exogenously added NANE did not serve as the substrate for desaturase, ruling out this possibility. Of the first two possibilities, the following line of evidence discounted the direct desaturation route and supported the quinone methide route: (i) the desaturase did not resemble other similar desaturases like succinate dehydrogenase or fatty acid desaturase, which are membrane bound and channel the two hydrogen atoms removed from their substrate into electron transport system; (ii) phenylthiourea, a specific inhibitor of phenoloxidases, drastically inhibited the dehydro-NADA formation, indicating the requirement of phenoloxidase component for desaturase action; (iii) HPLC analysis of the reaction mixture on HPLC system I (fig.2) usually yielded a minor peak at 2.17 min, which was identified to be NADA quinone; (iv) when the desaturase reaction was carried out in the presence of *N*-acetylcysteine (a powerful quinone trap), formation of both NANE and dehydro-NADA was totally inhibited

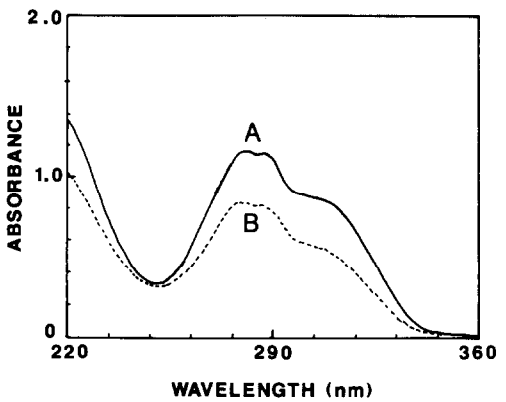

Fig.3. Ultraviolet spectrum of (A) authentic and (B) isolated dehydro-NADA in 0.2 M acetic acid.

(fig.4). At the same time, a new product was formed in the reaction which was identified to be *N*-acetylcysteinyl-NADA quinone adduct (fig.4).

From the foregoing discussion, it is clear that NADA quinone is an obligatory intermediate for the desaturation of the side chain of NADA. Since NADA quinone isomerase activity was present in

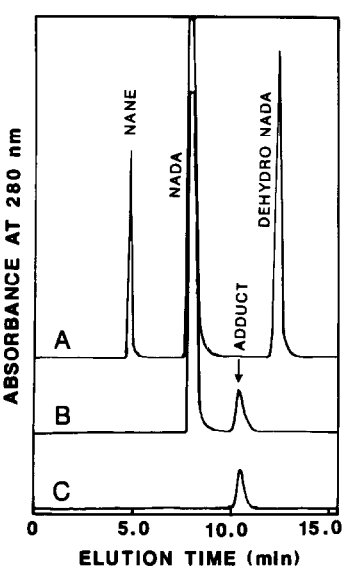

Fig.4. Trapping of transiently formed NADA quinone. The reaction conditions are the same as outlined for fig.1. HPLC system II was used. (A) Standard assay, (B) standard assay plus 2 mM *N*-acetylcysteine, (C) authentic *N*-acetylcysteinyl-NADA quinone adduct. The peaks at 4.95, 8.06, 10.66 and 12.65 min are due to NANE, unused NADA, *N*-acetylcysteinyl NADA-quinone adduct and dehydro-NADA, respectively.

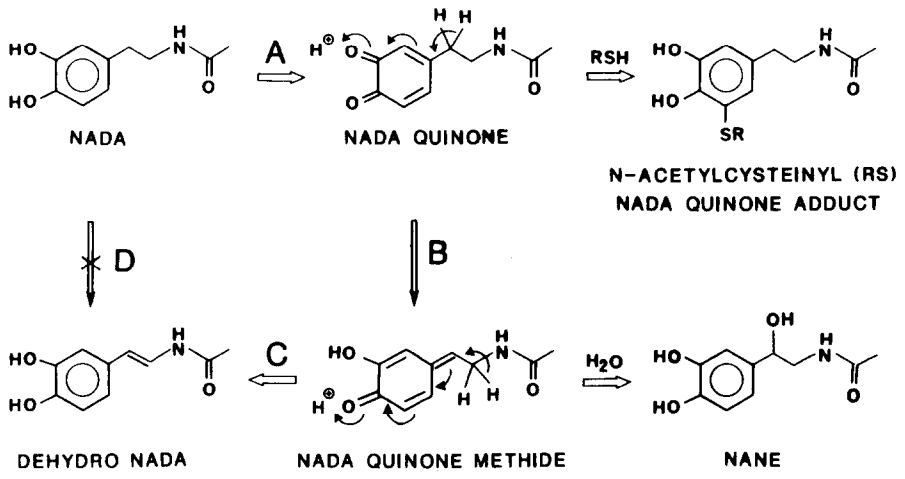

Fig.5. Proposed mechanism for the observed reactions. *N*-Acetyldopamine is oxidized by phenoloxidase (A) to NADA quinone. NADA quinone:NADA quinone methide isomerase (B) converts the NADA quinone to NADA quinone methide, which can either undergo non-enzymatic hydration to form NANE as the stable product or undergo enzyme catalyzed tautomerization (NADA quinone methide:dehydro-NADA isomerase); (C) to dehydro-NADA. In the presence of exogenously added *N*-acetylcysteine (RSH), NADA quinone formed is trapped as *N*-acetylcysteinyl-NADA quinone adduct, which prevents the two isomerase actions. Direct conversion of NADA to dehydro-NADA (D) is not observed.

the desaturase preparation, it is possible that NADA quinone methide formed from the NADA quinone could undergo non-enzymatic tautomerization to dehydro-NADA [3,12,13]. However, with the hemolymph NADA quinone isomerase, which is devoid of any desaturase activity [10], we did not observe any fortuitous formation of dehydro-NADA during the conversion of NADA quinone to NADA quinone methide. These studies indicate the presence of a separate isomerase which converts NADA quinone methide to dehydro-NADA. Due to the inability to generate NADA quinone methide chemically, it was not possible to test the activity of this isomerase directly. However, when hemolymph NADA quinone isomerase was exogenously added to desaturase reaction mixture, an increased amount of dehydro-NADA formation was observed (due to the availability of more NADA quinone methide for the second isomerase). Currently, we are attempting to resolve the NADA quinone methide:dehydro-NADA isomerase from NADA quinone:NADA quinone methide isomerase to show conclusively its existence. Nevertheless, the results presented in this communication clearly indicate: (i) the presence of a new enzyme system that desaturates the side chain of catecholamine derivative, NADA, and (ii) obligatory formation of NADA quinone and NADA quinone methide as intermediates of NADA conversion to dehydro-NADA.

Dehydro-NADA is one of the sclerotizing precursors for tanning of insect cuticle and a novel compound which exhibits interesting oxidation properties [3–5,11–13]. The discovery of the desaturase enzyme system must prove useful not only for understanding the biochemistry of cuticle sclerotization but also for the future development of new insect control measures, as sclerotization is a vital process for the survival of most insects [3,4,12,13].

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