Volume 255, number 2, 340–344 FEB 07629 September 1989

N-acetyldopamine quinone methide/1,2-dehydro-*N*-acetyl dopamine tautomerase

A new enzyme involved in sclerotization of insect cuticle

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Received 29 July 1989

The enzyme system causing the side chain desaturation of the sclerotizing precursor, *N*-acetyldopamine (NADA), was solubilized from the larval cuticle of *Sarcophaga bullata* and resolved into three components. The first enzyme, phenoloxidase, catalyzed conversion of NADA to NADA quinone and provided it for the second enzyme (NADA quinone isomerase), which makes the highly unstable NADA quinone methide. Quinone methide was hydrated rapidly and nonenzymatically to form *N*-acetylnorepinephrine. In addition, it also served as the substrate for the last enzyme, quinone methide tautomerase, which converted it to 1,2-dehydro-NADA. Reconstitution of NADA side chain desaturase activity was achieved by mixing the last enzyme fraction with NADA quinone isomerase, obtained from the hemolymph of the same organism, and mushroom tyrosinase. Therefore, NADA side chain desaturation observed in insects is caused by the combined action of three enzymes rather than the action of a single specific NADA desaturase, as previously thought.

Quinone methide; Catecholamine; Side chain desaturation; Cuticular sclerotization; *Sarcophaga bullata*; Quinone isomerase; Quinone methide isomerase

1. INTRODUCTION

At the conclusion of each molting cycle, the newly synthesized, soft and pale cuticle of several insects is hardened by sclerotization reactions to protect their soft bodies [1]. During this process, catecholamine derivatives such as *N*-acetyldopamine and *N*-β-alanyldopamine are oxidatively activated to form sclerotizing agents which are subsequently believed to react nonenzymatically with cuticular components, accounting for the hardening process [2–4]. Two types of sclerotizing modes have been discovered so far; one in which the aromatic ring of catecholamine derivatives participates in the crosslinking process (quinone tanning) and the other in which the aliphatic side chain of catecholamine contributes for the loci of macromolecular addition (β-sclerotization) [2–4]. In 1982, Andersen's group isolated dehydro NADA and invoked the quinone of this compound to be the sclerotizing agent for the latter process [3,5,6]. In the following year, we discovered quinone methides as a new type of sclerotizing agents and argued that quinone methides and not dehydro NADA quinone was the sclerotizing agent for the β-sclerotization process [2,4,7–10]. In addition, we chemically synthesized dehydro NADA for the first time [11,12] and, after examining its oxidation chemistry, concluded that the quinone methide derivative of dehydro NADA (or the corresponding radical) is the sclerotizing agent derived from this compound [13,14].

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

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

In spite of its key role in sclerotization, the mode of biosynthesis of dehydro NADA remained controversial. While Andersen insists that it is generated directly from NADA by a desaturase action (reaction D, fig.1) [3,5,6], we presented arguments in favor of our hypothesis that dehydro NADA is formed by an indirect route involving intermediary formation of NADA quinone and NADA quinone methide in insect cuticle (reactions A → B → C, fig.1) [4,8–10]. Yet realizing that the ultimate proof for our proposal can come only by demonstrating the enzyme activities responsible for such conversion, we attempted to solubilize the cuticular enzyme(s) concerned with the introduction of a double bond in the side chain of NADA. Our attempts led to the solubilization of an enzyme system from the larval cuticle of *Sarcophaga bullata* which catalyzed the conversion of NADA to dehydro NADA via NADA quinone [19]. Direct proof for the participation of NADA quinone methide in this reaction could not be obtained due to its high reactivity and our inability to chemically synthesize this compound. We have now resolved the components of the enzyme system and demonstrate in this communication that NADA quinone methide and not NADA is the direct precursor of dehydro NADA.

2. MATERIALS AND METHODS

2.1. *Enzyme preparation*

All operations were carried out at 0–5°C unless stated otherwise. Larvae of *Sarcophaga bullata* were raised on a dog food diet at 32°C. At the wandering stage, 160 g of larvae were collected, washed with distilled water and homogenized in a Waring blender for 30 s with water. The cuticle sheaths recovered by filtering the homogenate through a 100 μm screen, was rehomogenized for an additional 1 min in water and filtered. The cuticle was suspended in 0.1% sodium borate buffer, pH 8.5, for 2 h. The proteins released into the solution were recovered by centrifugation at 27000 × *g* for 30 min. The clear supernatant was designated as crude desaturase preparation and was loaded on a benzamidine Sepharose column (2.5 × 7.5 cm) previously equilibrated with 0.1% sodium borate buffer, pH 8.5. After washing the column extensively with the same buffer followed by 25 mM Tris-HCl, pH 7.5, bound proteins were eluted with 25 mM Tris-HCl, pH 7.5, containing 0.2 M sodium chloride. Fractions exhibiting quinone isomerase activity were pooled, desalted on a Sephadex G-25 column and lyophilized. The lyophilisate was taken up in minimum amounts of 25 mM Tris-HCl, pH 7.5, containing 0.2 M sodium chloride and chromatographed on a Sephacryl S-200 column (1.5 × 115 cm) equilibrated with the same buffer. Fractions of 1.8 ml were collected and assayed for both NADA quinone isomerase and NADA quinone methide tautomerase activities.

2.2. *Assay of enzyme activities*

Quinone isomerase was assayed using a 1-ml reaction mixture containing 20 μg mushroom tyrosinase and 1 mM NADA in 50 mM sodium phosphate buffer, pH 6.0. After NADA quinone formation was completed, an aliquot of quinone

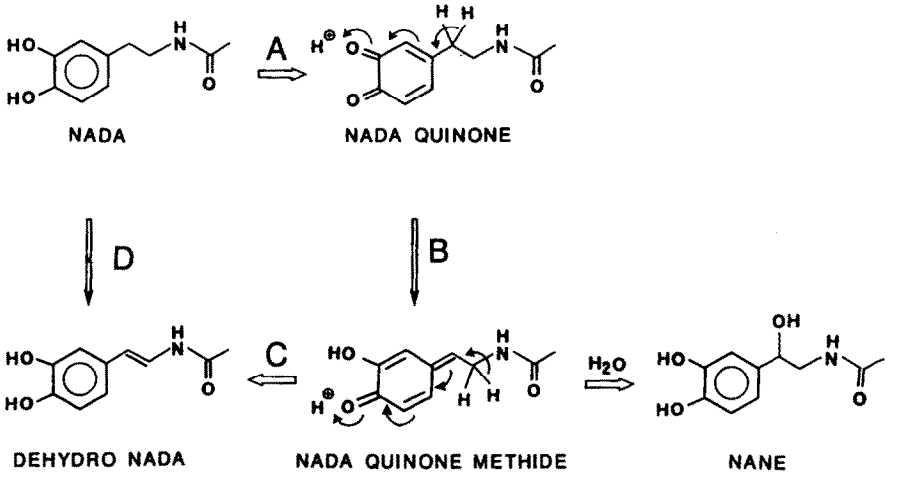

Fig.1. Two routes for the biosynthesis of dehydro NADA. According to Andersen [3,5,6], dehydro NADA is formed by a direct route involving NADA desaturation (reaction D). Our group favors the indirect route (reaction A → B → C) for achieving the NADA side chain oxidation [4,8–10]. Note that NADA quinone methide is converted to NANE by a nonenzymatic route involving a 1,6-addition of water. A, phenoloxidase; B, NADA quinone/quinone isomerase; C, NADA quinone methide/dehydro NADA tautomerase; D, NADA desaturase.

isomerase was added to the reaction mixture and the decrease in absorbance at 410 nm was monitored in a spectrophotometer. One unit of isomerase activity was defined as that amount of enzyme which catalyzes 50% decrease in the absorbance at 410 nm per min in the above reaction.

Quinone methide/dehydro NADA tautomerase was assayed using a reaction mixture (1 ml) containing 2 mM NADA, 30 μg mushroom tyrosinase and 0.3 U of quinone isomerase in 50 mM sodium phosphate buffer, pH 5.5. The reaction mixture was incubated at room temperature for 2.5 min and then analyzed for the formation of dehydro NADA by HPLC.

2.3. *HPLC analysis*

HPLC analysis of reaction mixtures was performed using a Beckman (Berkeley, CA) model 332 liquid chromatograph system equipped with two model 110B pumps, a model 420 controller, a model 160 absorbance detector and a model 427 integrator. Separations were carried out on a Beckman C_{18} ultrasphere reversed-phase column (5 μm, 4.6 × 150 mm) using an isocratic solvent system consisting of 50 mM acetic acid containing 0.2 mM sodium octyl sulfonate in 30% methanol at a flow rate of 0.6 ml/min.

2.4. *Other procedures*

Mushroom tyrosinase (spec. act. ≈4000 U/mg) and NADA were obtained from Sigma (St. Louis, MO). Dehydro NADA was synthesized as outlined earlier [11,12]. NANE was obtained by acetylation of norepinephrine using the published procedure [12]. Purification of quinone isomerase from the larval hemolymph of *S. bullata* has been described elsewhere [15]. Ultraviolet and visible spectra were recorded using a Gilford model 2600 spectrophotometer. These studies indicated the likely operation of an indirect route involving NADA quinone and NADA quinone methide in the biosynthesis of dehydro NADA.

3. RESULTS AND DISCUSSION

The crude desaturase preparation obtained from the larval cuticle of *S. bullata* readily converted NADA to NANE and dehydro NADA. Earlier, we had demonstrated that NANE is generated by the nonenzymatic hydration of NADA quinone methide formed by the combined action of phenoloxidase/quinone isomerase on NADA [15–18]. Accordingly, the crude desaturase preparation contained both phenoloxidase and NADA quinone isomerase activities. The dehydro NADA formation by the desaturase preparation was dependent on the presence of phenoloxidase activity and was completely inhibited by 2 μM phenylthiourea, a specific inhibitor of phenoloxidase. Furthermore, when NADA quinone was prepared by silver oxide oxidation and provided to the desaturase preparation, it readily generated both NANE and dehydro NADA, confirming the obligatory requirement of NADA quinone for both NANE and dehydro NADA formation.

According to our proposal, NADA quinone methide is the immediate precursor for dehydro NADA (see fig.1). But, due to its very high reactivity and our inability to chemically synthesize this compound, we could not directly check this possibility. However, when quinone isomerase from hemolymph, which is devoid of any desaturase activity, was added to the crude desaturase preparation, increased dehydro NADA formation (due to increased supply of the substrate, NADA quinone methide) could be observed, indicating the presence of a separate NADA quinone methide/dehydro NADA tautomerase activity in the desaturase preparation. To confirm the presence of this enzyme, we resolved the components of desaturase system as outlined in section 2. The phenoloxidase, due to its sticky nature, was not chromatographed but bound irreversibly to the benzamidine column and lost its activity. Both quinone isomerase and quinone methide/dehydro NADA tautomerase could be obtained and resolved into individual components on Sephacryl S-200 column chromatography (fig.2).

Peak I (fig.2) readily transformed the enzymatically generated NADA quinone to NANE, confirming that this peak is due to quinone

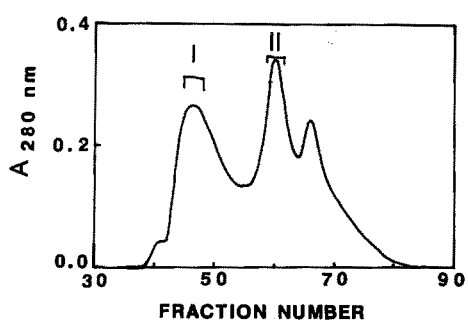

Fig.2. Sephacryl S-200 chromatography of a partially purified NADA desaturase preparation from *S. bullata* larvae. The active fractions from a benzamidine Sepharose column (see section 2) were chromatographed on a Sephacryl S-200 column (1.5 × 115 cm) equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl. Fractions of 1.8 ml were collected and assayed for both quinone isomerase and quinone methide tautomerase as described in section 2. Peak I contained quinone isomerase activity while peak II contained quinone methide tautomerase activity.

isomerase activity. Peak II isolated from the Sephacryl S-200 column (fig.2), did not oxidize NADA (reaction A, fig.3) or attack NADA quinone generated by the action of mushroom tyrosinase (reaction B, fig.3). However, when it was mixed with mushroom tyrosinase and quinone isomerase (reaction C, fig.3), it readily converted NADA (4.3 min peak) to dehydro NADA (6.1 min peak). Heat-inactivated enzyme did not support this conversion (reaction D, fig.3). NANE formation observed in reactions C and D was due to the nonenzymatic addition of water molecules on enzymatically generated NADA quinone methide by quinone isomerase. The 6.1 min peak observed in fig.3C is due to dehydro NADA and was confirmed by comparison of HPLC retention times, cochromatography and UV spectroscopy (fig.4). That the tautomerization of NADA quinone methide is not of nonenzymatic origin can be demonstrated by the following studies. As shown in fig.3D, addition of heat-inactivated peak II to a reaction mixture containing NADA, quinone isomerase and mushroom tyrosinase failed to support the synthesis of dehydro NADA. Moreover, NADA quinone isomerase alone failed to synthesize any detectable dehydro NADA. Typical enzyme kinetic studies could not be carried out on quinone methide tautomerase due to our inability to chemically synthesize NADA quinone methide,

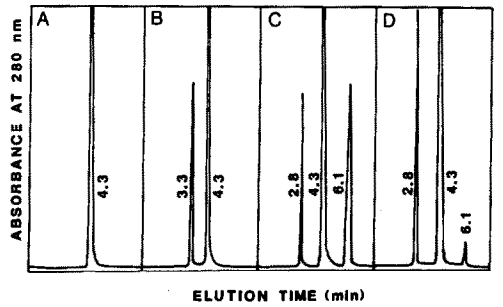

Fig.3. Reconstitution of NADA desaturase activity. A reaction mixture containing 2 mM NADA and quinone methide tautomerase (peak II of Sephacryl column; 100 μl) in 50 mM phosphate buffer (pH 5.5) was incubated at room temperature and 20-μl aliquots were subjected to HPLC analysis as outlined in section 2. (A) 5-min reaction. (B) 5-min reaction which also contained 30 μg mushroom tyrosinase. (C) B + quinone isomerase (0.3 units) for 2.5 min. (D) Heat-denatured quinone methide tautomerase in place of native enzyme. The peaks at 2.8, 3.3, 4.3 and 6.1 min are due to NANE, NADA quinone, NADA and dehydro NADA, respectively.

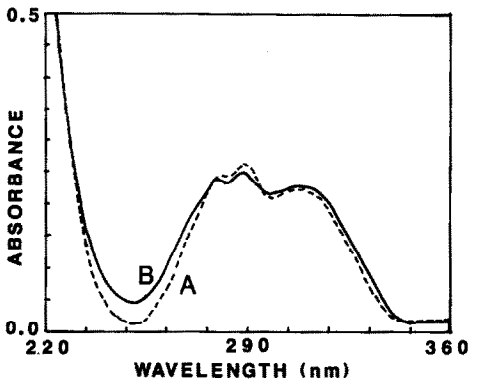

Fig.4. UV spectrum of: (A) authentic dehydro NADA and (B) enzymatic product (peak 6.1 from fig.3C).

but, along with phenoloxidase and quinone isomerase, the quinone methide isomerase exhibited a pH optimum of 5.5. Currently, we are engaged in the further study of these enzyme components in order to shed more light on this novel enzymatic reaction.

The results presented in this communication, therefore, confirm our hypothesis that dehydro NADA is biosynthesized by the indirect route (fig.1) involving NADA quinone and NADA quinone methide [4,8–10] and do not support the direct desaturase route (reaction D, fig.1) advocated by Andersen [3,5,6].

Acknowledgements: This work was supported by a grant from the National Institutes of Health (RO1-AI-14753).

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