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Trapping of transiently formed quinone methide during enzymatic conversion of *N*-acetyldopamine to *N*-acetylnorepinephrine

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We have demonstrated that quinone methide formation is an important aspect of insect physiology and proposed that enzymatically generated quinone methides react nonenzymatically with water or other nucleophiles to form Michael-1,6-addition products [(1988) Adv. Insect Physiol. 21, 179–231; (1989) J. Cell. Biochem. suppl. 13C, 58]. Using a purified *o*-quinone isomerase from the larval cuticle of *Sacrophaga bullata* and mushroom tyrosinase, we now demonstrate that transiently formed *N*-acetyldopamine quinone methide from *N*-acetyldopamine can be trapped by methanol to produce *β*-methoxy *N*-acetyldopamine. The methanol adduct thus formed was found to be a racemic mixture and can be resolved into the optical isomers on cyclodextrin chiral column. These results confirm our contention that enzymatically generated quinone methides are nonenzymatically and nonstereoselectively transformed to Michael-1,6-adducts by reaction with water or other nucleophiles.

Cuticular tanning; Sclerotization; Catecholamine metabolism; Phenoloxidase; Quinone isomerase; Quinone methide; (*Sarcophaga bullata*)

1. INTRODUCTION

The transformation of soft and pale cuticle into hard and tanned exoskeleton observed soon after ecdysis in most insects is due to sclerotization reactions [1]. During this process, soluble structural proteins and chitin fibers are rendered insoluble and inextractable from cuticle by crosslinking reactions involving reactive sclerotizing agents generated by the enzymic oxidation of *N*-acyldopamine derivatives such as NADA and *N*-*β*-alanyldopamine [2–4]. Based on the reactive species generated, two types of sclerotizing modes have been established. These are quinone tanning, discovered by Pryor [5,6] and quinone methide sclerotization, discovered by our group [2–4,7–11]. Initially, we proposed a direct route for the generation of quinone methides from 4-alkylcatechols [7–11]. However, recent studies from our laboratory reveal that quinone methides are not directly generated from 4-alkylcatechols but are indirectly produced by the combined action of *o*-diphenoloxidase and a novel *o*-quinone:*p*-quinone methide isomerase in insect cuticle [12–14]. According to our mechanism, sclerotizing precursors such as NADA and *N*-*β*-alanyldopamine are oxidized by cuticular phenoloxidase to their corresponding quinones. *o*-Quinone:*p*-quinone methide isomerase converts the phenoloxidase-generated quinones to quinone methides. Quinone methides thus formed react nonenzymatically with cuticular nucleophiles to form cuticle-catecholamine adducts or react with water to form *N*-acylnorepinephrines [2–4,7–14]. Accordingly, we have observed both adduct formation with cuticle and generation of side chain hydroxylated catecholamine derivatives during cuticular enzyme-mediated oxidation of catecholamine derivatives [2–4,7,9–14].

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Abbreviations: NADA, *N*-acetyldopamine; NANE, *N*-acetylnorepinephrine

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We have also isolated a soluble *o*-quinone:*p*-quinone methide isomerase from the hemolymph of *Sarcophaga bullata* which readily tautomerizes phenoloxidase generated 4-alkylquinones to 2-hydroxy-*p*-quinone methides [15]. Due to the high reactivity of the simple quinone methide derivatives, it was not possible to directly identify them. Therefore, the question of whether quinone methides generated in the reaction are enzymatically hydrated or nonenzymatically converted to side chain hydroxylated products remains to be answered. In the hope of answering this question, we have conducted some trapping experiments with a soluble enzyme system that generates quinone methides; the results re-affirm our earlier contention that quinone methides are nonenzymatically and nonstereoselectively converted to Michael-1,6-addition products.

2. MATERIALS AND METHODS

2.1. *Cuticular enzyme preparation*

All operations were carried out at 0 to 5°C unless stated otherwise. Larvae of *Sarcophaga bullata* were raised on a dog food diet at 37°C. At wandering stage they were collected, washed with distilled water and homogenized in ice-cold water with a Virtis Omnimixer at maximum speed for 30 s. The homogenate was passed through a 100 μm screen and the recovered cuticle was re-homogenized for an additional 2 min with ice-cold water. The cuticle was recovered by sieving and suspended in 200 ml of 0.1 M sodium borate buffer, pH 8.5, for 2 h. The contents were passed through two layers of cheese cloth and the filtrate was saturated to 30% with ammonium sulfate. After centrifugation at 10000 × *g* for 10 min, the clear supernatant obtained was recovered and brought up to 60% saturation with respect to ammonium sulfate. The precipitated proteins were collected by centrifugation, dissolved in 15% ammonium sulfate and loaded onto a phenyl-Sepharose column (2.5 × 5 cm) equilibrated with the same solution. After washing the column with about 200 ml of this solution, quinone isomerase activity was eluted with 25 mM Tris-HCl buffer, pH 7.5. The active fractions were lyophilized, desalted on a Sephadex G-25 column and then loaded on a DEAE-Sepharose column (1 × 5 cm) equilibrated with 25 mM Tris-HCl buffer, pH 7.5. After washing, bound proteins were eluted with the same buffer containing 100 mM NaCl. The eluate was concentrated by lyophilization and chromatographed on a Sephacryl S-200 column (1.5 × 110 cm) using 25 mM Tris-HCl containing 0.2 M NaCl. Fractions containing the isomerase activity were pooled and used as the enzyme source.

2.2. *HPLC analysis*

HPLC analysis of reaction mixture was performed as outlined in our earlier publication [12]. Resolution of racemic mixture of β-methoxy-NADA was carried out on an acetylated β-cyclodextrin (acetylated cyclobond I) column (5 μm, 4.6 × 250 mm) using isocratic elution with 20% methanol containing 0.1% triethylamine adjusted to pH 4.0 with acetic acid at a flow rate of 1 ml/min.

Mushroom tyrosinase (spec. act. 4000 U/mg) and NADA were obtained from Sigma (St. Louis, MO). NANE was synthesized from norepinephrine by acetylation [16]. 6-Methoxydopamine was prepared by deblocking the protective group from *N*-carbobenzyloxy-6-methoxydopamine kindly provided by Professor Bernard Witkop of National Institutes of Health.

3. RESULTS AND DISCUSSION

The quinone isomerase isolated from *Sarcophaga bullata* larval cuticle by the procedure outlined in section 2 did not possess any *o*-diphenoloxidase activity towards catechols such as NADA (fig.1, trace A), but readily converted exogenously provided NADA quinone (prepared by oxidation of NADA either enzymatically with mushroom tyrosinase or chemically with silver oxide) to NANE. Fig.1, trace B, shows the conversion of tyrosinase-generated NADA quinone to NANE. The production of NANE can occur in two ways; viz., the NADA quinone methide formed in the reaction may be either enzymatically hydrated or nonenzymatically transformed to give the observed product. Our earlier studies while supporting the occurrence of the nonenzymatic route [2–4,9,12–14], did not unequivocally rule out the former mechanism. In order to differentiate between these two routes, we conducted the following experiments.

If NADA quinone methide is freely generated from the active site of quinone isomerase, we should be able to trap it with a suitable nucleophile. Accordingly we made numerous attempts to trap this transient intermediate with *N*-acetylcysteine. However, all our attempts met with failure, as *N*-acetylcysteine, being a powerful quinone trap, rapidly trapped the NADA quinone and prevented its availability to quinone isomerase. Our attempts to trap the quinone methide with weak nucleophiles such as acetate or phosphate also ended in failure. However, when the reaction was conducted in 10% methanol, we did observe the formation of a new compound (fig.1 trace C, 15.7 min peak). This product was absent in control reactions in which quinone isomerase was omitted indicating that it is a methanol adduct of NADA quinone methide. Since the synthetic analog was not readily available

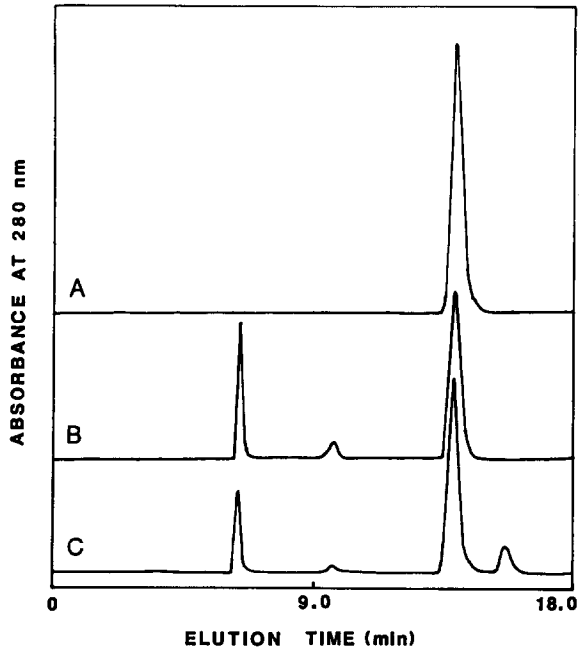

Fig.1. HPLC analysis of the NADA-mushroom tyrosinase-quinone isomerase reaction. (A) A reaction mixture containing 600 μM NADA, isomerase in 50 mM sodium phosphate, pH 6.0, was incubated at room temperature for 10 min and a 20 μl aliquot was subjected to HPLC analysis on a Beckman C_{18}-IP ultrasphere reversed-phase column (5 μm, 4.6 × 250 mm). Isocratic elution with 50 mM acetic acid containing 0.2 mM sodium octylsulfonate in 20% methanol was used as the solvent at a flow rate of 0.6 ml/min. (B) Same as above but the reaction contained 20 μg of mushroom tyrosinase. (C) Same as A but the reaction contained 20 μg of mushroom tyrosinase and was carried out in the presence of 10% methanol. The peaks eluting at 6.36 min, 9.58 min, 13.84 min and 15.7 min are due to NANE, NADA-quinone, NADA and β-methoxy NADA, respectively.

we could not identify this compound by comparison. However, UV spectral studies readily attested to the identity of this compound. The UV absorbance maximum of this compound (λ_{max} = 279 nm) corresponded with that of NANE (λ_{max} = 278 nm; water adduct of NADA quinone methide) and differed significantly from 6-methoxydopamine (λ_{max} = 289 nm; methanol adduct of dopamine quinone) and 5-*S*-(*N*-acetyl) cysteinyl NADA (λ_{max} = 254 and 290 nm; *N*-acetylcysteine adduct of NADA quinone) (see fig.2). Based on these studies the adduct was tentatively identified to be the β-methoxy NADA. Formation of NANE and β-methoxy NADA is consistent with the liberation

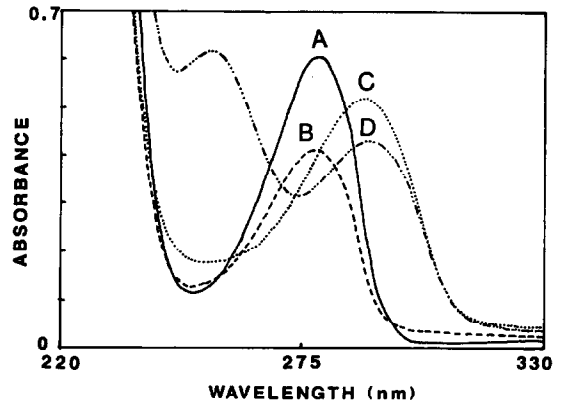

Fig.2. Ultraviolet absorbance spectra of substituted catecholamines. The spectra were recorded in 0.2 M acetic acid. (A) β-Methoxy NADA; (B) NANE; (C) 6-methoxydopamine; (D) 5-*S*-(*N*-acetyl)cysteinyl-NADA adduct.

of NADA quinone methide and its subsequent nonenzymatic reactions with water and methanol. If this is true, then we should observe the reaction to be nonstereoselective as well. Hence we attempted to resolve the NANE and β-methoxy NADA formed on chiral columns. Although our attempts to resolve the NANE formed in the reaction were unsuccessful, we could separate the β-methoxy NADA on acetylated cyclodextrin column (fig.3) confirming our contention the nonenzymatic and

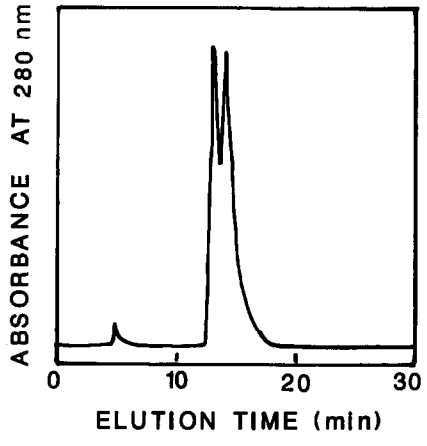

Fig.3. Resolution of β-methoxy NADA formed in the reaction mixture. β-Methoxy NADA formed in the enzymatic reaction (fig.1, trace C, 15.7 min peak) gave a single symmetrical peak in different HPLC systems. However, on HPLC analysis on the chiral cyclobond I column, the racemic mixture was resolved into the two optical isomers (13.0 and 14.0 min peaks).

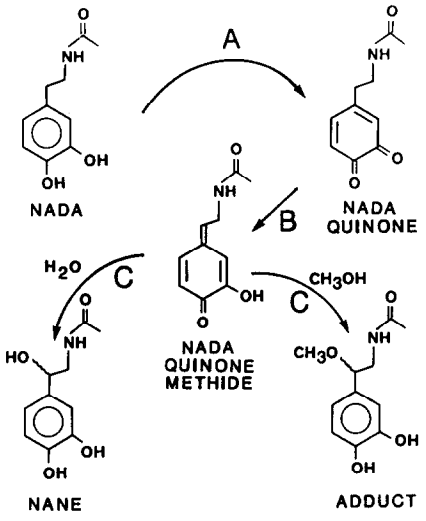

Fig.4. Summary of the observed reactions. NADA is oxidized by tyrosinase (A) to NADA quinone which is acted upon by *o*-quinone-*p*-quinone methide isomerase (B) to generate NADA quinone methide. NADA quinone methide thus formed, being very unstable, undergoes rapid nonenzymatic hydration to NANE or reacts with methanol to form *β*-methoxy NADA (C, nonenzymatic transformations).

nonstereoselective nature of NANE formation and *β*-methoxy NADA formation from NADA quinone methide.

The scheme shown in fig.4 accounts for the observed reactions. Tyrosinase oxidizes NADA to its quinone which is isomerized to NADA quinone methide by quinone isomerase. The NADA quinone methide thus formed reacts with water to produce NANE or, with methanol, to generate *β*-methoxy NADA. Since the reactions of NADA quinone methide are nonenzymatic they are also nonstereoselective as observed in the case of *β*-methoxy NADA. Quinone methide formation from catecholamine derivatives was originally proposed by Witkop and his associates as a possible mechanism for the biosynthesis of norepinephrine type of compounds as early as 1958 [17–19]. Our studies not only reconfirm this classical work but also revitalize it as a key process in insect physiology.

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REFERENCES

[1] Chapman, D.F. (1982) The Insects: Structure and Function, pp.501–528, Harvard University Press, Cambridge.
[2] Sugumaran, M. (1987) in: Molecular Entomology. UCLA Symposia on Molecular and Cellular Biology, New Series, vol.49 (Law, J.A. ed.) pp.357–367, A.R. Liss, New York.
[3] Sugumaran, M. (1987) Bioorg. Chem. 15, 194–211.
[4] Sugumaran, M. (1988) Adv. Insect Physiol. 21, 179–231.
[5] Pryor, M.G.M. (1940) Proc. R. Soc. London Ser. B 128, 378–392.
[6] Pryor, M.G.M. (1940) Proc. R. Soc. London Ser. B 128, 393–407.
[7] Sugumaran, M. and Lipke, H. (1982) FEBS Lett. 155, 65–68.
[8] Lipke, H., Sugumaran, M. and Henzel, W. (1983) Adv. Insect Physiol. 17, 1–84.
[9] Sugumaran, M. (1988) Arch. Insect Biochem. Physiol. 8, 73–88.
[10] Sugumaran, M., Hennigan, B., Semensi, V. and Dali, H. (1988) Arch. Insect Biochem. Physiol. 8, 89–100.
[11] Sugumaran, M., Hennigan, B., Semensi, V., Mitchell, W. and Rivera, T. (1988) Arch. Insect Biochem. Physiol. 8, 229–241.
[12] Saul, S.J. and Sugumaran, M. (1988) FEBS Lett. 237, 155–158.
[13] Sugumaran, M., Saul, S.J. and Semensi, V. (1988) Arch. Insect Biochem. Physiol. 9, 269–281.
[14] Sugumaran, M., Semensi, V. and Saul, S.J. (1989) Arch. Insect Biochem. Physiol. 10, 13–27.
[15] Sugumaran, M. (1989) J. Cell. Biochem. Suppl. 13C, 58.
[16] Dali, H. and Sugumaran, M. (1988) Org. Prep. Proc. Int. 20, 191–195.
[17] Senoh, S., Witkop, B., Creveling, C.R. and Udenfriend, S. (1958) IV Inter. Congress of Biochem., vol.XIII, pp.176–188, Pergamon, London.
[18] Senoh, S. and Witkop, B. (1959) J. Am. Chem. Soc. 81, 6222–6231.
[19] Senoh, S., Creveling, C.R., Udenfriend, S. and Witkop, B. (1959) J. Am. Chem. Soc. 81, 6236–6240.