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Nonenzymatic transformations of enzymatically generated *N*-acetyldopamine quinone and isomeric dihydrocaffeiyl methyl amide quinone

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We have recently demonstrated that the side chain hydroxylation of *N*-acetyldopamine and related compounds observed in several insects is caused by a two-enzyme system catalyzing the initial oxidation of catecholamine derivatives and subsequent isomerization of the resultant quinones to isomeric quinone methides, which undergo rapid nonenzymatic hydration to yield the observed products [Saul, S.J. and Sugumaran, M. (1989) FEBS Lett. 249, 155–158]. During our studies on *o*-quinone/*p*-quinone methide tautomerase, we observed that quinone methides are also produced nonenzymatically slowly, under physiological conditions. The quinone methide derived from *N*-acetyldopamine was hydrated to yield *N*-acetylnorepinephrine as the stable product as originally shown by Senoh and Witkop [(1959) J. Am. Chem. Soc. 81, 6222–6231], while the isomeric quinone methide from dihydrocaffeiyl methylamide exhibited a new reaction to form caffeiyl amide as the stable product. The identity of this product was established by UV and IR spectral studies and by chemical synthesis. We could not find any evidence of intramolecular cyclization of *N*-acetyldopamine quinone to iminochrome-type compound(s). The importance of quinone methides in these reactions is discussed.

Quinone methide; Quinone isomerase; Side chain desaturation; Sclerotization; Quinone imine; Catecholamine metabolism

1. INTRODUCTION

The key role of catecholamine derivatives such as *N*-acetyldopamine and *N*-β-alanyldopamine in sclerotization of insect cuticle is well established [1–4]. Since sclerotization is an extremely important biochemical process for the survival of most insects, the oxidation chemistry of *N*-acetyldopamine derivatives forms an important aspect of cuticular biochemistry. During sclerotization, the catecholamine derivatives are enzymatically activated to sclerotizing agents, which are then believed to react with cuticular components nonenzymatically forming adducts and crosslinks in the hardening process. Depending on the sclerotizing agents generated, two types of sclerotizing modes have been established – quinone tanning, proposed originally by Pryor [5,6] and quinone methide sclerotization, discovered by our group [1–4,7–11]. While quinones undergo a Michael-1,4-addition reaction with available nucleophiles in cuticle, quinone methides exhibit 1,6-addition reactions to form catecholamine adducts with cuticular nucleophiles, accounting for the hardening process [1–4]. Although we initially proposed a direct two electron oxidation route for the generation of quinone methides [1–4,7,8], recent work from our laboratory indicates that quinone methides are not produced directly from the catecholamine derivatives but indirectly from the phenoloxidase-generated quinones by tautomerization reaction [9–11]. Although tautomerization of quinones to quinone methides can occur even nonenzymatically, as observed in the case of carboxymethyl-*o*-

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Abbreviations: CMA, caffeiyl methylamide; DHCMA, dihydroxycaffeiyl methylamide; NADA, *N*-acetyldopamine; NANE, *N*-acetylnorepinephrine

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benzoquinone [12], we did identify an enzyme mediating this reaction in insect cuticle [9–11]. Furthermore, we have purified a soluble *o*-quinone/*p*-quinone methide isomerase from the hemolymph of *Sarcophaga bullata* to about 99%, which may be involved in insect immunity in addition to its key role in sclerotization [13,14]. During our studies on this newly discovered isomerase, we observed nonenzymatic formation of quinone methides from NADA and isomeric DHCMA; the details of this study are presented in this paper.

2. MATERIALS AND METHODS

2.1. *Materials*

Mushroom tyrosinase (spec. act. ≈4000 U/mg protein), NADA, dihydrocaffeic acid and caffeic acid were obtained from Sigma Chemical Co., St. Louis, MO. Visible spectral studies were carried out using a Gilford model 2600 spectrophotometer. IR spectra were recorded in a Perkin Elmer model 137 spectrophotometer. ^{1}H-NMR spectra were recorded using a 60 MHz Perkin-Elmer model R-24 spectrometer. NADA-quinone isomerase from *Sarcophaga bullata* was purified as outlined in an earlier paper [14]. HPLC analyses were carried out as described in an earlier publication [9]. Separations were achieved on a Beckman ultrasphere ODS column (5 μm, 4.6 × 150 mm) using isocratic elution with 50 mM acetic acid containing 0.2 mM sodium octyl sulfonate in 20% methanol.

2.2. *Synthesis of caffeiyl methyl amide (CMA)*

A mixture of caffeic acid (1.8 g; 10 mmol) in *N,N*-dimethylformamide (5 ml), an excess of methylamine in tetrahydrofuran and dicyclohexylcarbodiimide (2.06 g; 10 mmol) was stirred at room temperature for 16 h. The reaction mixture was then filtered, diluted with water and extracted with ethyl acetate. The organic layer was recovered and dried over anhydrous magnesium sulfate. Removal of solvent on a rotary evaporator gave a red-colored oil; crystallization from methanol gave a buff-colored solid (0.9 g; yield, 47%), m.p. 226–227°C, single compound by HPLC. ^{1}H-NMR (DMSO-d_6): δ = 2.75 (doublet, changed to singlet on D_2O exchange, 3H, CH_3), 6.35 (doublet, J = 16 Hz, 1H, CH = CH), 6.70–7.20 (multiplet, 3H, ArH), 7.35 (doublet, J = 16 Hz, 1H, CH=CH), 7.75–8.15 (broad singlet, 1H, NH), 8.50–9.70 ppm (broad singlet, 2H, OH), NH and OH signals were exchanged with D_2O.

2.3. *Synthesis of dihydrocaffeiyl methyl amide (DHCMA)*

A mixture of dihydrocaffeic acid (1.82 g; 10 mmol), and excess of methylamine and dicyclohexylcarbodiimide (2.06 g; 10 mmol) was processed as above and chromatographed on a Biogel P-2 column using 0.2 M acetic acid as the solvent. The fractions containing the required product were lyophilized: white solid (0.5 g; yield, 26%), m.p. 84–85°C. ^{1}H-NMR (DMSO-d_6): δ = 2.10–2.50 (multiplet, 2H, CH_2), 2.55 (doublet, changed to singlet on D_2O exchange, 3H, CH_3), 2.60–2.90 (multiplet, 2H, CH_2), 6.25–6.80 (multiplet, 3H, ArH), 6.30 (broad singlet, 2H, OH), 7.20–7.90 ppm (broad singlet, 1H, NH), OH and NH signals were exchanged with D_2O.

3. RESULTS AND DISCUSSION

The stability of NADA quinone generated by mushroom tyrosinase-catalyzed oxidation of NADA is shown in fig.1 (trace A). As published earlier [14], it was found to be reasonably stable and exhibited slow transformation. However, when *o*-quinone/quinone methide isomerase isolated from *S. bullata* was added to this solution (trace B), the NADA quinone disappeared rapidly with the concomitant appearance of NANE.

Fig.1 (inset A) shows the HPLC analysis of the NADA-tyrosinase reaction mixture. From the figure, it is clear that traces of NANE are formed in the reaction containing NADA and tyrosinase only. For comparison, the HPLC analysis of NADA-tyrosinase-quinone isomerase is also given in the same figure (inset B). The NANE formation

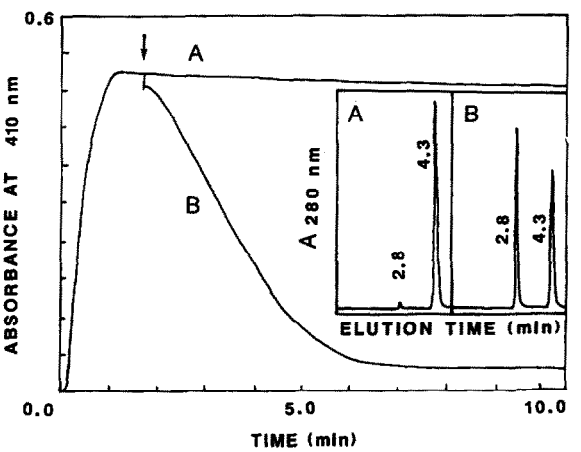

Fig.1. Nonenzymatic and enzymatic conversion of NADA-quinone to NANE. A reaction mixture containing 1 mM NADA, 20 μg mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature, and the stability of quinone formed in the reaction mixture was continuously monitored at 410 nm. (A) Without any additive. (B) With 0.15 U of NADA quinone isomerase. Inset: HPLC analysis of the reaction. The above reaction mixtures (10 μl aliquots) were mixed with ascorbic acid to reduce the quinones back to catechols and were subjected to HPLC analysis as outlined in section 2. A flow rate of 0.6 ml/min was maintained. (A) Phenoloxidase reaction; (B) Phenoloxidase/quinone isomerase reaction; peaks at 2.8 and 4.3 are due to NANE and NADA, respectively.

from NADA quinone in the latter case has been shown to occur via enzyme-mediated isomerization of NADA quinone to NADA quinone methide and its subsequent nonenzymatic hydration [9,10,14]. A similar but nonenzymatic route for the formation of norepinephrine derivatives as shown by Witkop and his associates [15,16] accounts for the formation of NANE in the reaction mixture lacking isomerase. Therefore, it was of interest to compare the rates of both nonenzymatic and enzymatic transformation.

Such comparisons revealed that NANE was produced from NADA quinone (via NADA quinone methide) generated by the mushroom tyrosinase (10 μg) catalyzed oxidation of 1 mM NADA, at a rate of about 1 nmol·min^{-1}·ml^{-1} of reaction mixture. Under identical conditions, inclusion of 1 U of quinone isomerase generated NANE at a rate of about 400 nmol·min^{-1}·ml^{-1}. Thus the enzymatic tautomerization of NADA quinone to its quinone methide is at least 400 times faster than the nonenzymatic reaction. We have demonstrated that quinone isomerase exhibits wide substrate specificity and attacks quinones of NADA, *N*-β-alanyldopamine and 3,4-dihydroxyphenethyl alcohol [9–11,14]. Hence, it was of interest to study the oxidation of DHCMA, which is isomeric with NADA.

Fig.2 illustrates the spectral changes associated with the oxidation of DHCMA by mushroom tyrosinase. Examination of visible spectral changes shows that the DHCMA quinone is formed but does not accumulate in the reaction mixture, although ultraviolet spectral changes continue to occur. This suggested to us that DHCMA quinone is unstable and is undergoing further reactions. In order to find out the product(s) formed in the reaction mixture, HPLC studies were carried out. HPLC analysis of the reaction shown in fig.3 confirmed this contention and indicated the appearance of new compounds. The minor peaks at 2.17 min and 6.02 min were not identified. The peak at 3.0 min was identified to be the DHCMA quinone, and the major product accumulating at 7.4 min was tentatively identified to be CMA, based on elution time and co-chromatography with

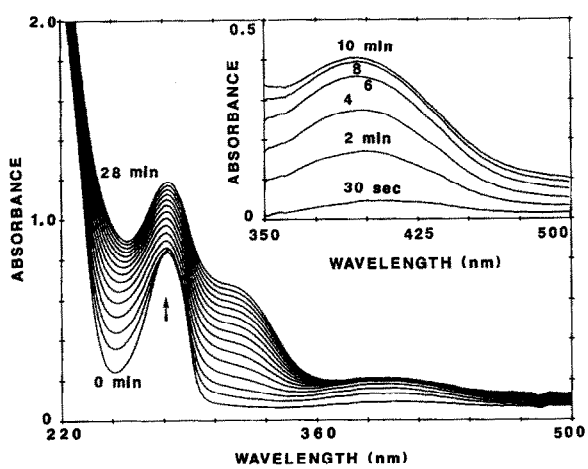

Fig.2. Spectral changes associated with the oxidation of DHCMA by mushroom tyrosinase. A reaction mixture containing 100 μg of DHCMA, 1 μg of mushroom tyrosinase in 1 ml of 100 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and the spectral changes accompanying the oxidation were monitored at 2-min time intervals. After 10 min, there was no increase in absorbance at the visible region, but spectral changes continued to occur in the UV region. Inset: visible spectral changes of the reaction during the initial phase of oxidation. Substrate and enzyme concentrations were doubled for this; the rest of the conditions were the same as above.

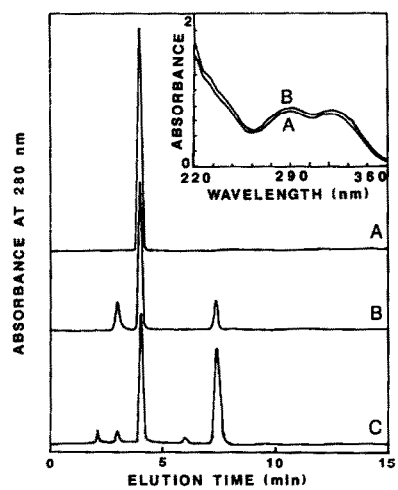

Fig.3. HPLC analysis of the DHCMA-tyrosinase reaction. A reaction mixture containing 100 μg of DHCMA, 0.5 μg mushroom tyrosinase in 1 ml of 100 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature, and an aliquot (15 μl) was subjected to HPLC analysis as outlined in section 2. A flow rate of 1 ml/min was used. The 3.0 min peak is due to the DHCMA quinone, the 4.0 min peak is due to DHCMA and the 7.4 min peak is due to CMA. (A) 0 time; (B) 12-min reaction; (C) 48-min reaction. Inset: the ultraviolet absorbance spectrum of: (A) isolated product (7.4 min peak) and (B) authentic CMA.

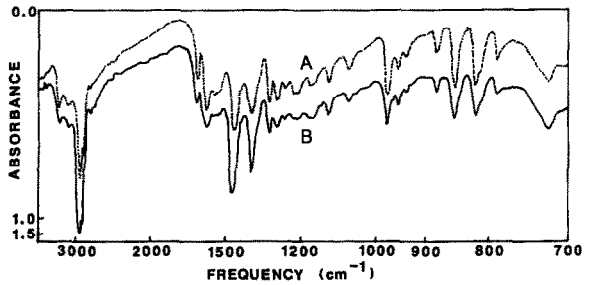

Fig.4. IR spectrum of caffeiyl methyl amide showing the IR spectrum of the: (A) enzymatic product and (B) authentic CMA.

the authentic sample. To unequivocally prove the structure of the product, a large-scale reaction was conducted and the product formed was isolated. Both the UV absorbance spectrum (fig.3, inset) and the IR spectrum (fig.4) of the product matched that of an authentic CMA, confirming that they are one and the same compound.

It is interesting to note that the DHCMA quinone methide and NADA quinone methide take two entirely different routes for their further nonenzymatic transformations. While the former undergoes a rapid prototropic transformation, the latter is simply hydrated to form NANE (fig.5). Perhaps the electron-withdrawing carbonyl group and the quinone methide make the methylene protons acidic and hence facilitate its removal in DHCMA quinone methide, while NADA quinone methide seems to be comparatively stable and undergoes an external nucleophilic addition reaction. Accordingly, we did find that even nucleophiles other than water, such as methanol, could add onto this reactive species nonenzymatically to form racemic β-methoxy NADA [24].

Transformation of quinone methide to a side chain desaturated catechol is an interesting reaction. We have earlier proposed a similar conversion for the synthesis of 1,2-dehydro-*N*-acetyldopamine from NADA in insect cuticle [2–4,8]. But, with the purified NADA quinone isomerase, we did not observe any such product formation [14]. However, we have shown the presence of a separate NADA quinone methide tautomerase carrying out this reaction from the larval cuticle of *S. bullata* [25].

In the case of DHCMA, the bulk of the quinone was converted to CMA, but with NADA we observed only about 2–3% conversion of the quinone to NANE nonenzymatically, which is also in agreement with the earlier work published by Peter [17]. Kramer et al. [18,19] have claimed that they observed the intramolecular cyclization of *N*-acetyldopamine quinones to an iminochrome-type compound similar to the dopa to dopachrome conversion during electrochemical and enzymatic oxidation of these compounds. We could not find

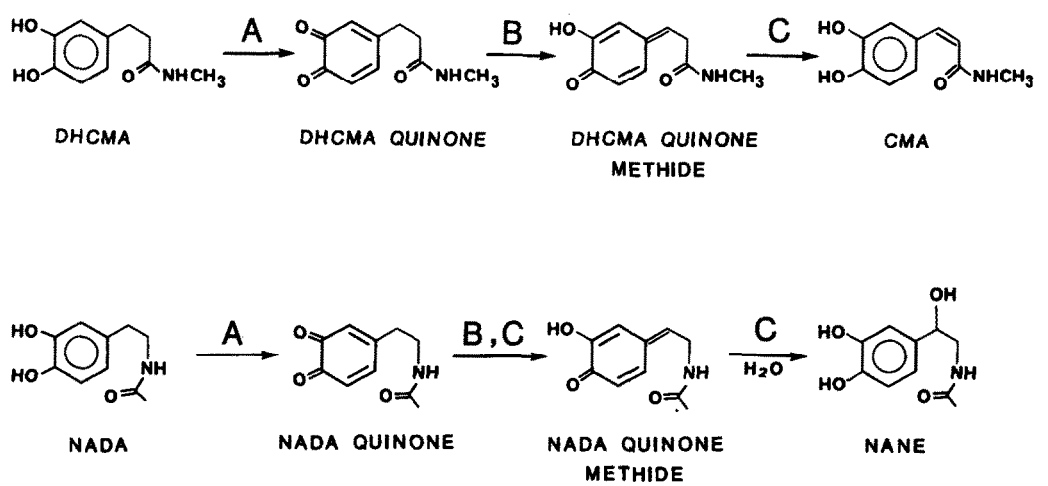

Fig.5. Proposed mechanism for the observed reactions. NADA quinone formed by enzymatic oxidation of NADA is either nonenzymatically or enzymatically converted to NANE, while the isomeric DHCMA quinone formed by enzymatic oxidation undergoes rapid tautomerization to CMA. (A) Phenoloxidase, (B) quinone isomerase, (C) nonenzymatic transformation.

any evidence for the formation of dopachrome-type compounds from NADA quinone, either spectrophotometrically or by HPLC. In light of the electronic structure of the amide bond, it is rather difficult to believe that such a reaction is possible, as amide nitrogen, due to electron delocalization, is positively charged and has apparently lost the nucleophilic properties of amines [20]. Accordingly, while studying the oxidation of NADA spectroscopically, Graham and Jeffs [21] pointed out that NADA quinone does not form a 'dopachrome'-type compound. Similarly, during ESR studies on the oxidation of NADA, two independent groups of investigators reported the lack of cyclization of NADA quinone [22,23]. Our studies also point to the same conclusion and are in agreement with the electronic structure and chemical behavior of NADA quinone. Since Kramer et al. [18,19] used crude enzyme extracts during their electrochemical studies, the possibility of NADA (or its quinone) getting hydrolyzed at a slow rate to dopamine (or its quinone) and subsequently converted to 'dopaminechrome' cannot be ruled out. Alternately, nonenzymatic Michael-1,4-addition of water to the *N*-acyldopamine quinone to form 6-hydroxy-*N*-acyldopamine derivatives as observed by Witkop's group [15,16] can also account for the electrochemical data presented by the authors. Since electrochemistry is not a structural elucidation technique, it is possible that these authors misinterpreted this information and believed that NADA quinone cyclized to form the leucochrome. Therefore, further studies are essential to confirm the possibility of such a reaction occurring with NADA quinone. Experiments in this direction are in progress in our laboratory.

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