Detection of Semiquinone Radicals of N-Acylcatecholamines in Aqueous Solution

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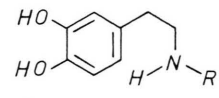
ESR-spectra were recorded during the oxidation of N-acetyldopamine and N-β-alanyldopamine in aqueous solutions. Semiquinone radicals were detected under conditions of spin stabilization by Zn^{2+} ions. The appearance of the spectra was the same in the presence or in the absence of proteins. No evidence was obtained for the formation of products that could have arisen eventually from intermolecular Michael-type addition of nitrogen nucleophiles.

N-Acetyldopamine and N-β-alanyldopamine are important natural products. They are involved in oxidative processes which lead to the formation of a sclerotized exoskeleton in insects.

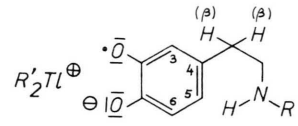
The addition of primary or secondary amines to *o*-quinones leads to 4-amino-1,2-dihydroxybenzene derivatives [1]. This reaction is well known in organic chemistry, and it is thought to occur as well in several biologically important systems where *o*-quinones are involved in the denaturation of proteins or in the formation of melanin polymers. By far the best understood example is the intramolecular cyclization of 3,4-dihydroxyphenethylamine derivatives which leads to leucoaminochromes as intermediates in polymerization. It is questionable, however, whether analogous products are formed also when there is no possibility for an intramolecular attack of an aliphatic amino group. Such a situation would be represented by the oxidation of N-acylcatecholamines which in many insects are essential constituents of the sclerotized exoskeleton [2]. It is thought that cuticle proteins are crosslinked by the corresponding *o*-quinones *via* aminoquinoneimines. However, competing reactions may occur, such as polymerization of semiquinone radicals [3] or desamination of protein side chain amino groups [4].

Intermediates in the oxidative intramolecular cyclization of 3,4-dihydroxyphenethylamines have been recently characterized under physiological conditions by ESR spectroscopy, using the spin stabilization effect of divalent metal cations [5]. We have used a similar approach in order to see whether Michael-type addition products may occur as intermediates in the denaturation of proteins by *o*-quinones. Thus, the ESR-spectra of several spin stabilized amino substituted semiquinones were investigated in a previous study [6]. We now report on the ESR-spectra of semiquinone radicals of N-(3,4-dihydroxyphenethyl)-acetamide (N-acetyldopamine) and 3-amino-N-(3,4-dihydroxyphenethyl)-propionamide (N-β-alanyldopamine). Spectra of reaction mixtures containing bovine serum albumin or the insect larval serum protein manducin [7] were also recorded.



$R = -\overset{O}{\overset{\|}{C}}-CH_3$ N-ACETYLDOPAMINE

$R = -\overset{O}{\overset{\|}{C}}-CH_2-CH_2-NH_2$ N-β-ALANYLDOPAMINE



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Materials and Methods

ESR-spectroscopy

See [6].

Substrates and proteins

N-Acetyldopamine and N-β-alanyldopamine were synthesized as described previously [7]. BSA was purchased from Sigma (Cat. No. A-4503). The insect larval serum protein manducin was prepared from haemolymph of 5–7 days old 5th instar *Manduca sexta* larvae as described previously [7]. Mushroom tyrosinase (monophenol monooxygenase, EC 1.14.18.1) was from Serva (Cat. No. 37619). ESR-spectra of fully sclerotized insect cuticles were recorded from shed exuviae of *Manduca sexta* pupae (*cf.* [8]).

Oxidation of N-acylcatecholamines

Reaction mixtures contained either 20 mM N-acetyldopamine or 20 mM N-β-alanyldopamine in 50 mM potassium phosphate buffer pH 6.8 or in 3 mM $ZnSO_4$ pH 5.5. Methyl β-alanate was used at a concentration of 20 mM. The concentrations of proteins were 4 $mg \cdot ml^{-1}$ BSA or 1.25 $mg \cdot ml^{-1}$ manducin. Tyrosinase was used at a concentration of 0.2 $mg \cdot ml^{-1}$, corresponding to ca. 280 $U \cdot ml^{-1}$. Appropriate mixing of undegased stock solutions of the individual components was followed by immediate recording of an ESR-spectrum and subsequently repeated scans after time intervals of 4 to 30 min.

Results

ESR-spectra of semiquinones of monocyclic amino substituted catechols show clearly a nitrogen coupling of ca. 2.0 G which increases remarkably in the corresponding indole type derivatives [6]. The ESR-spectra of the diorganothallium complexes of the N-acetyldopamine semiquinone radicals in pyridine, *cf.* Fig. 1, show clearly the coupling with all α- and β-protons. The splitting constants are assigned for the diethylthallium counterion as $a_{H(3)} = 0.55$; $a_{H(5)} = 3.65$; $a_{H(6)} = 1.05$ and $a_{H(\beta)} = 3.00$ G with a line width of $\Delta H = 0.25$ G. The *g*-value is 2.00408 relative to 4-*tert*-butoxy-2,6-di-*tert*-butylphenoxy radical ($g = 2.00463$). Essentially the same spectrum is obtained from the corresponding thallium complexed dopamine semiquinone radical in pyridine [9]. Changing the solvent to H_2O and the counterion

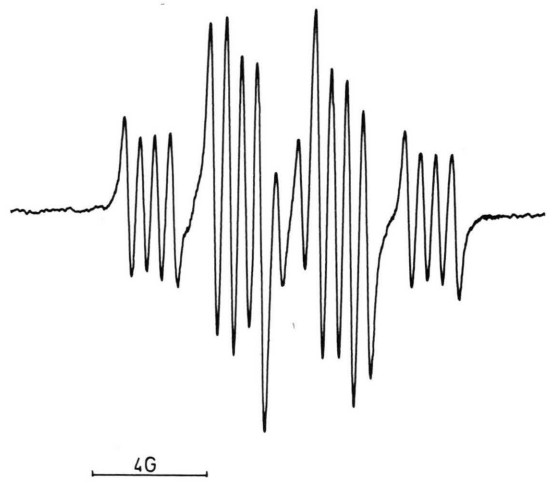

Fig. 1. ESR-spectrum (high field part) of N-Acetyldopamine semiquinone radical complex with diethyl thallium hydroxide in pyridine.

to Zn^{2+} the hyperfine structure of the dopamine semiquinone remains unchanged with slightly different coupling constants [10] whereas in the ESR-spectra of N-acyldopamine semiquinones the smallest coupling could not be resolved. For this reason we studied the solvent dependence of the N-acetyldopamine radical very briefly. We found an increase of $a_{H(5)}$ and $a_{H(\beta)}$ whereas $a_{H(6)}$ and $a_{H(3)}$ became smaller with increasing water concentration. On this basis we can assign the N-acetyldopamine semiquinone hyperfine structure in H_2O/Zn^{2+} as $a_{H(5)} = 4.00$; $a_{H(6)} = 0.63$, and $a^{(2)}_{H(\beta)} = 3.25$ G.

When N-acetyldopamine is oxidized with tyrosinase in phosphate buffer, a weak ESR-signal can be detected after 3 min, and the solution aquires a brown colour. The appearance of the spectrum does not allow any structural assignments. However, typical semiquinone radical spectra are observed after mixing aqueous solutions of the N-acylcatecholamines and $ZnSO_4$. Since no enzyme is required, these radicals are formed by autoxidation. The concentration increases in time, reaching a maximum after 30 to 60 min. No major differences in semiquinone concentration were observed in the presence of tyrosinase, though the oxidation was accelerated as revealed by the brown colour of these solutions. The presence of primary amines, either in form of peptidic lysine ε-amino groups, or as methyl β-alanate, or as in N-β-alanyldopamine, does not lead to any observable differences in the ESR-spec-

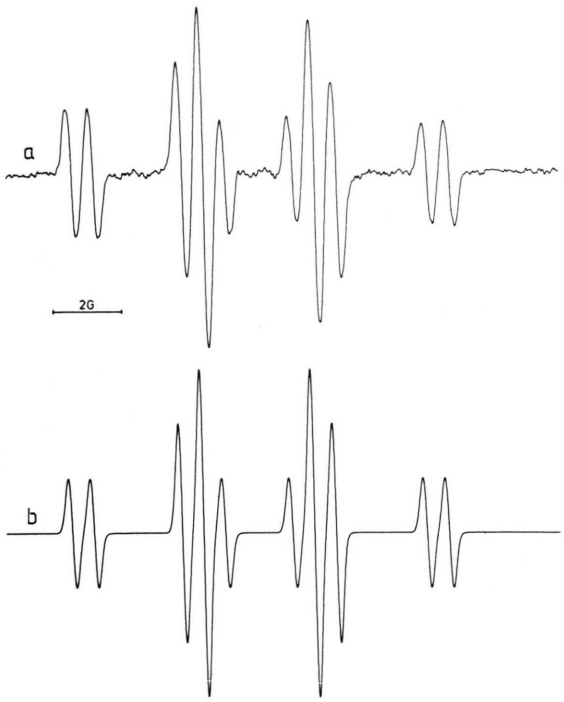

Fig. 2. a) ESR-spectrum of a mixture of 20 mM N-β-alanyldopamine, 1.25 mg · ml^{-1} manducin, and 0.2 mg · ml^{-1} tyrosinase in 3 mM $ZnSO_4$. The spectrum was recorded 3 min after mixing of the components;
b) Simulated ESR-spectrum of catechol semiquinone radical, using $a_{H(5)} = 3.85$; $a_{H(6)} = 0.625$; $a_{H(\beta)} = 3.20$ G, and a line width of $\triangle H = 0.2$ G.

tra. Fig. 2 shows the ESR-spectrum of a reaction mixture containing N-β-alanyldopamine in addition to tyrosinase and the insect larval serum protein manducin which is a component of the cuticle sclerotization system in *Manduca sexta* [11]. A computer simulation of the spectrum, in which proton hyperfine couplings $a_{H(5)} = 3.85$; $a_{H(6)} = 0.625$, and $a_{H(\beta)} = 3.20$ G, and a line width $\Delta H = 0.2$ G was used, is also included in Fig. 2.

Prolonged standing of the solutions results in a decrease of the intensity of the signals, indicating slow secondary reactions. There was, however, in no case evidence for the formation of aminosemiquinones (*cf.* [5, 6]). Most likely, the intermediate semiquinones polymerize by oxidative coupling processes. When an ESR-spectrum is recorded of oxidation products of N-acetyldopamine that had been chromatographically separated from low M_r components [12], the appearance of an unresolvable complex signal is indicative for the presence of polymerized phenolic materials (Fig. 3). Sclerotized insect cuticle which was obtained from exuviae of *Manduca sexta* pupae, shows a broad structureless ESR-signal which is typical for melanin-type polymers, as has been observed before by Mason *et al.* [13].

Discussion

The results of the oxidation of N-acetyldopamine and N-β-alanyldopamine in aqueous solutions under the conditions of spin stabilization reveal that semiquinone radicals may occur as intermediates in the oxidation of insect cuticle sclerotization agents. Insect cuticle contains laccase and peroxidase in addition to tyrosinase [14, 15] (see also [16] for review). Though tyrosinase is thought to catalyze two-electron oxidations only [17], the generation of semiquinone radicals in insect cuticle may well occur through the action of the other enzymes.

In the present investigation, no evidence was obtained for the occurrence of Michael-type additions

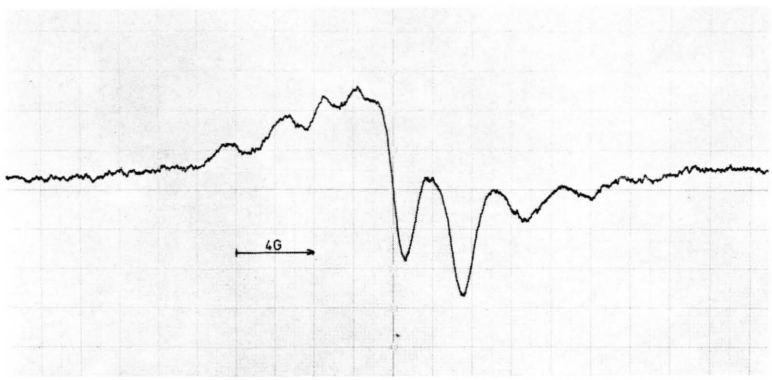

Fig. 3. ESR-spectrum of oxidation products of N-acetyldopamine. Tyrosinase was added to a solution containing 33 mM N-acetyldopamine and 33 mM β-alanine methyl ester in 50 mM KPO_4 buffer pH 6.8. After 48 h incubation at 22 °C, the reaction mixture was resolved by chromatography on a Sephadex LH-20 column. Reaction products eluting immediately behind the salt fraction (*cf.* [12]) were subjected to ESR-spectroscopy.

of amines to the *o*-quinones or semiquinones generated from N-acylcatecholamines under physiological conditions. This result supports our earlier conclusion, namely that during insect cuticle sclerotization by N-acetyldopamine and N-β-alanyldopamine, crosslinking of cuticle proteins is not effected by aminoquinoneimines [8].

Instead, noncovalent interactions between a catechol-type melanin (see [18] for definition) and cuticle proteins, in addition to copolymerization of tyrosine residues with semiquinones, seems to be the more reasonable alternative [8, 11]. Noncovalent complexes between oxidation products of N-acetyldopamine and chitin have also been observed [19].

The principal structure of the polymers derived from N-acetyldopamine and N-β-alanyldopamine are presently unknown. Kramer *et al.* [20] have described a slow intramolecular cyclization of N-acylcatecholamines which should lead to leukoaminochromes during an electrochemical or enzymatic oxidation. Since corresponding semiquinone radicals were not detected in the present study, it is unlikely that they do indeed occur physiologically to any significant amount.

Acknowledgement

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