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Catalytic Oxidation of 2-Aminophenols and Ortho Hydroxylation of Aromatic Amines by Tyrosinase†

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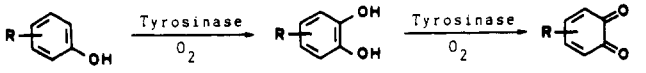
ABSTRACT: The usual substrates of tyrosinase, a copper-containing monooxygenase (EC 1.14.18.1), are monophenols and *o*-diphenols which are both converted to *o*-quinones. In this paper, we studied the reaction of this enzyme with two new classes of substrates: aromatic amines and *o*-aminophenols, structural analogues of monophenols and *o*-diphenols, respectively. They undergo the same catalytic reactions (ortho hydroxylation and oxidation), as documented by product analysis and kinetic studies. In the presence of tyrosinase, arylamines and *o*-aminophenols are converted to *o*-quinone imines, which are isolated as quinone anils or phenoxazones. As an example, in the presence of tyrosinase, 2-amino-3-hydroxybenzoic acid (an *o*-aminophenol) is converted to cinnabarinic acid, a well-known phenoxazone, while *p*-aminotoluene (an aromatic amine) gives rise to the formation of 5-amino-2-methyl-1,4-benzoquinone 1-(4-methylanil). Kinetic studies using an oxygen electrode show that arylamines and the corresponding monophenols exhibit similar Michaelis constants (K_m = 0.11–0.49 mM). In contrast, the reaction rates observed for aromatic amines are relatively slow (k_{cat} = 1–3 min^{-1}) as compared to monophenols (1320–6960 min^{-1}). The enzymatic conversion of arylamines by tyrosinase is different from the typical ones: N-oxidation and ring hydroxylation without further oxidation. This difference originates from the regiospecific hydroxylation (ortho position) and subsequent oxidation of the intermediate *o*-aminophenol to the corresponding *o*-quinone imine. Finally, the well-known monooxygenase activity of tyrosinase was also confirmed for the aromatic amine *p*-aminotoluene, with $^{18}O_2$. In the case of *o*-aminophenols, the kinetic studies indicate that the K_m values are rather similar to those of the corresponding *o*-diphenols. The oxidation rates, k_{cat}, for *o*-aminophenols are comparable to 2,3-dihydroxybenzoic acid (0.2–0.8 s^{-1}).

Tyrosinase is a copper-containing monooxygenase (EC 1.14.18.1) that catalyzes the ortho hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mason, 1957) (Scheme I). This enzyme is widely distributed in nature and is responsible for the formation of melanin pigments and other polyphenolic compounds (Lerch, 1981).

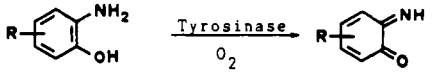
The active site of tyrosinase consists of a binuclear copper center where activation of molecular oxygen and substrate interaction take place (Schoot Uiterkamp & Mason, 1973; Lerch, 1983; Wilcox et al., 1985). As anticipated from the chemical knowledge of copper-catalyzed hydroxylations and oxidations (Nigh, 1973; Capdevielle & Maumy, 1987), the enzymatic activity of tyrosinase should not be only restricted to aromatic hydroxy compounds (monophenols and catechols).

It seemed therefore of interest to study the activity of tyrosinase on aromatic amines and *o*-aminophenols, which are analogues of monophenols and *o*-diphenols, respectively. These compounds are widespread in nature and have been shown to be metabolized under various conditions (Tomoda et al., 1984).

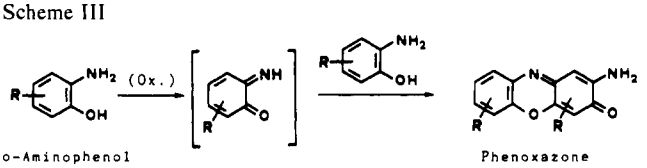
Scheme I



Scheme II



Scheme III



First, we observed that *o*-aminophenols undergo an oxidation reaction in the presence of tyrosinase leading to the formation of the corresponding *o*-quinone imines (Scheme II).

Different oxidizing agents, manganese dioxide (Prinz & Savage, 1977) and potassium ferricyanide or dichromate (Schäfer, 1964), and some metalloproteins, hemoglobin (To-

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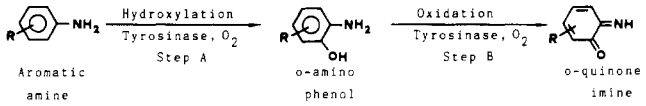
Scheme IV



moda, 1984) and catalase (Ogawa et al., 1983a,b), for example, can perform this reaction. The products of the reaction are not the *o*-quinone imines themselves, as they are highly reactive, but in most cases derivatives such as phenoxazones (Grünanger, 1979) (Scheme III). The phenoxazone ring occurs in many pigments; moreover, it is also found in antibiotics, e.g., actinomycin (Schäfer, 1964).

Prompted by these findings, we decided to study also the reaction of tyrosinase with arylamines (the structural analogues of monophenols). Arylamines have been the subject of a great deal of work as many of these substances are potentially carcinogenic (Clayson & Gardner, 1976). They are metabolized via two main types of oxidation processes: N-oxidation (the amino group is hydroxylated leading to hydroxylamine derivatives further oxidized to nitroso compounds) and ring hydroxylation (the aromatic ring of arylamines is hydroxylated in the ortho or para position). Typical enzymatic systems responsible for the N-oxidation are, for example, hepatic cytochrome P-450 and flavin-containing monooxygenases (Frederick et al., 1982) or catalase (Böttcher & Kiese, 1960) while ring hydroxylations are catalyzed by the xanthine oxidase/hypoxanthine system (Radzik et al., 1983), the *p*-hydroxybenzoate hydroxylase (Entsch et al., 1976), and oxy- and methemoglobin (Guillochon et al., 1984).

In this study, we show that arylamines are substrates of tyrosinase undergoing an ortho hydroxylation like the corresponding monophenols (Scheme IV, step A). In contrast to *p*-hydroxybenzoate hydroxylase, however, the resulting *o*-aminophenol is not released but further oxidized to the corresponding *o*-quinone imine (Scheme IV, step B).

Materials and Methods

Neurospora crassa tyrosinase was prepared according to Lerch (1987). Kinetic constants were determined with a Yellow Springs Instruments oxygen monitor (Model 53) and an oxygen probe (5331). Labeled oxygen ($^{18}O_2$, 98%) was obtained from the Cambridge Isotope Laboratories, Woburn, MA. Mass spectra were acquired on a Varian 711 and the ^{1}H NMR spectra on a Varian XL-200. The chemical shifts in $CDCl_3$ or DMSO-d_6 are relative to internal tetramethylsilane.

Oxygen Uptake Measurements. The cells were filled with 3 cm^3 of air-saturated substrate, dissolved in 0.1 M potassium phosphate, pH 7. After temperature equilibration (~3 min), tyrosinase was injected (0.8 nmol for the kinetic study of arylamines and 1.4 nmol in the case of *o*-aminophenols). The rate of oxygen uptake was calculated from the linear portion of the recorded curve. The kinetic constants were obtained from Eadie–Scatchard and Hanes–Woolf plots.

Reaction of Tyrosinase with 3-Hydroxyanthranilic Acid (**1**). At 24 °C, *N. crassa* tyrosinase (17 nmol) is added to a stirred solution of **1** (76.5 mg, 0.5 mmol) in 100 cm^3 of 0.1 M potassium phosphate buffer, pH 7.0. After 4 h, the mixture is allowed to settle overnight in order to achieve the precipitation of the product. The crystals, obtained by centrifugation, are washed with water and ethanol and then placed in refluxing ethanol for 1 h. After cooling and centrifugation, the red crystals of cinnabarinic acid are dried: yield, 34 mg (45%) (not optimized); TLC analysis, silica gel; eluant, butanol/acetic acid/water (4:2:1); mass spectrometry (EI) (Varian 711, 214 °C) 300 (M^+, 0.6), 256 ($M^+ - CO_2$, 19), 212 ($M^+ - 2CO_2$, 98), 44 (CO_2, 100); UV–visible spectroscopy (EtOH) λ_{max} 443, 425, 233 nm; ^{1}H NMR (200 MHz, DMSO-d_6) δ 6.6 (1 H), 7.55–7.95 (3 H), 8.8 (1 H), 9.7 (1 H), 13.7 (1 H), 15.3 (1 H).

Table I: Oxidation of Various Aminophenols and Parent Compounds by *N. crassa* Tyrosinase[a]

benzoic acid	K_m (mM) (±0.1)	k_{cat} (s^{-1})
2-amino-3-hydroxy	0.7	0.25 ± 0.01
3-amino-2-hydroxy	11.3	0.40 ± 0.01
3-amino-4-hydroxy	4.5	0.83 ± 0.01
4-amino-3-hydroxy	4.4	0.60 ± 0.01
3,4-diamino	2.1	0.23 ± 0.01
2,3-dihydroxy	3.3	0.18 ± 0.01
3,4-dihydroxy	2.7	51.4 ± 0.2

[a] Conditions: 0.1 M potassium phosphate buffer, pH 7 (3 cm^3); substrate concentration range 0.24–24 mM; temperature 24 °C.

Reaction of Tyrosinase with p-Aminotoluene. To a gently stirred solution of *p*-aminotoluene (53.5 mg, 0.5 mmol) in 0.1 M potassium phosphate, pH 7 (15 cm^3), was added tyrosinase (40 nmol) at room temperature. The product formed precipitated as dark purple crystals. After 24 h, the reaction mixture was centrifugated and the supernatant, containing the unreacted starting material, was discarded. The crystals were washed twice with water, quickly rinsed with ethanol, and dried. An alternative treatment was purification by preparative thin-layer chromatography [eluant cyclohexane/ethyl acetate (1:1)]. By product analysis, the isolated substance was found to be a quinone anil, 5-amino-2-methyl-1,4-benzoquinone 1-(4-methylanil): mp 148.5 °C [lit. (Horner & Sturm, 1955) mp 148 °C]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.3 (3 H), 2.4 (3 H), 4.4 (2 H), 5.9 (1 H), 6.5 (1 H), 6.75 (2 H), 7.2 (2 H); mass spectrometry (average values from six scans) 224 (4.46) (M − 2), 225 (68.3) (M − 1), 226 (100) (M), 227 (19.1) (M + 1), 228 (11.8) (M + 2); UV–visible spectroscopy λ_{max} (EtOH) 474 and 289 nm [lit. (Horner & Sturm, 1955) 471 and 295 nm]. The yield was only 36% owing to the slow rate of the reaction (see Table I). On smaller scales, nearly quantitative yields were obtained by increasing either the reaction time or the ratio enzyme/substrate.

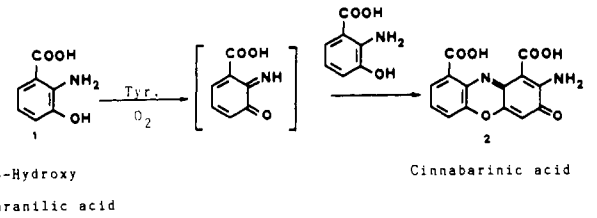
^{18}O-Labeling Experiment. In the flask containing $^{18}O_2$ (250 cm^3), a deaerated solution of *p*-aminotoluene (53.5 mg, 0.5 mmol) in 0.1 M potassium phosphate, pH 7.0 (15 cm^3), was introduced through a septum. Tyrosinase (40 nmol) was then injected and the flask slowly shaken for 6 h. The mixture was immediately centrifugated (in order to minimize the formation of the product in air) and treated as above to give about 3 mg of the quinone anil, which was analyzed by mass spectrometry. For the molecular peak region the following values were found: 226 (47.9), 227 (71.9), and 228 (100).

Results

Oxidation of o-Aminophenols. We have studied the reaction of a number of disubstituted benzoic acids (2-amino-3-hydroxy, 3-amino-2-hydroxy, 3-amino-4-hydroxy, 4-amino-3-hydroxy, 3,4-diamino, 2,3-dihydroxy, 3,4-dihydroxy) with tyrosinase. The kinetic constants (K_m and k_{cat}) have been determined with an oxygen electrode, while the chemical transformation of the substrates was studied by product analysis.

The uptake of oxygen during the reaction was measured at 24 °C. The results are summarized in Table I. The reaction of tyrosinase with 2-amino-3-hydroxybenzoic acid or 3-hydroxyanthranilic acid (**1**) (Scheme V) can be easily followed either by thin-layer chromatography or UV–visible spectroscopy.

Scheme V



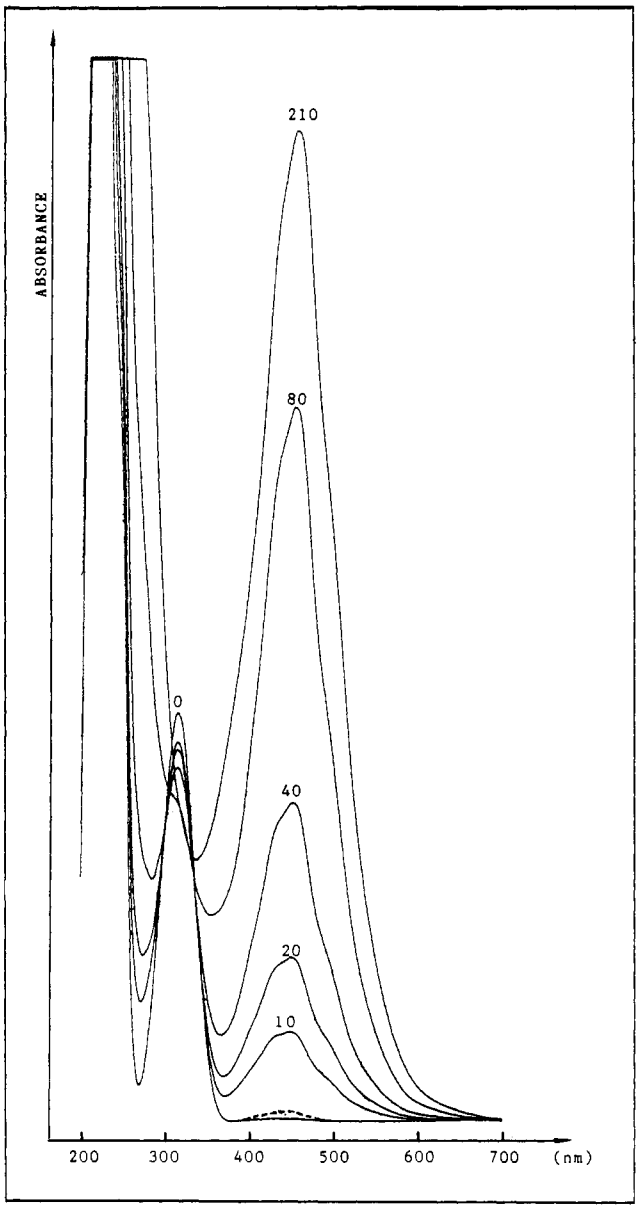


FIGURE 1: UV–visible spectra of the oxidation of 3-hydroxyanthranilic acid by *N. crassa* tyrosinase. Conditions: 0.1 M potassium phosphate, pH 7.0 (3 cm^3); substrate 3 mM; enzyme 0.8 nmol; reaction time in minutes. The broken line shows a control experiment. Conditions as above but without enzyme; incubation time 6 h.

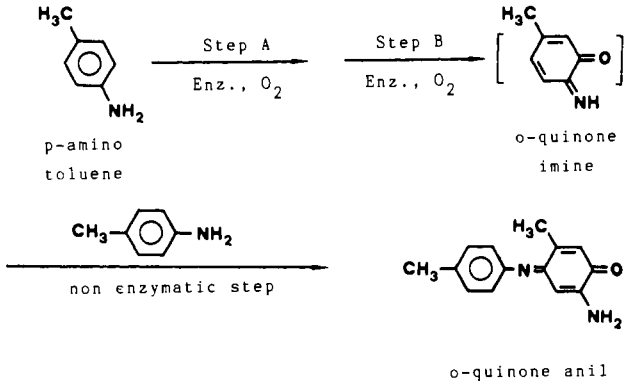
Samples were removed at different intervals and scanned between 200 and 700 nm. At 314 nm, the disappearance of the substrate **1** can be followed as well as the formation of the product **2** at 449 nm (Figure 1). Under the conditions mentioned in Figure 1, the reaction is almost quantitative after 2 h at 24 °C. Control experiments (without tyrosinase, broken line in Figure 1) showed that less than 0.5% of the substrate **1** is oxidized by air after 6 h. The product **2** formed during the enzymatic reaction has been identified as cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) in a preparative run (Scheme IV). An authentic sample of cinnabarinic acid was prepared according to Prinz and Savage (1977). All chemical and spectral features of the product formed during the enzymatic reaction and of the authentic cinnabarinic acid were found to be identical (thin-layer chromatography, UV–visible spectra, mass spectra, and ^1H NMR); see Materials and Methods.

Table II: Kinetic Constants of the Hydroxylation of Amino or Hydroxy Aromatic Derivatives by *N. crassa* Tyrosinase[a]

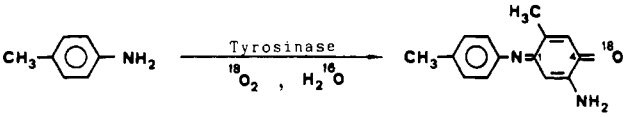
substrate	K_m (mM) (±0.02)	k_{cat} (min^{-1})
4-aminophenylalanine	0.37	3 ± 0.1
4-hydroxyphenylalanine (tyrosine)	0.34	6960 ± 70
4-aminotoluene (*p*-toluidine)	0.11	1.5 ± 0.1
4-hydroxytoluene (*p*-cresol)	0.13	2520 ± 30
4-aminoacetanilide	0.18	2 ± 0.1
4-hydroxyacetanilide	0.49	1320 ± 50

[a] For conditions, see Table I.

Scheme VI



Scheme VII



Hydroxylation of Arylamines. The reaction of tyrosinase with arylamines has been studied both by kinetic and product analysis. The kinetic constants obtained by oxygen uptake measurements are summarized in Table II. For comparative purposes, also the corresponding hydroxy derivatives (monophenols) were considered.

To establish the nature of the chemical transformation of the arylamines in the presence of tyrosinase, a product analysis was carried out. When *p*-aminotoluene was incubated with tyrosinase, a major product was formed (a quinone anil, see Scheme VI) while only traces of other compounds could be detected by thin-layer chromatography (Materials and Methods).

The same quinone anil is obtained from *p*-aminotoluene with Fremy's salt, a hydroxylating and oxidizing reagent (Horner & Sturm, 1955).

To confirm the monooxygenase activity of tyrosinase, established earlier for monophenols (Mason et al., 1955), with arylamines as substrates, we carried out a labeling experiment using $^{18}O_2$ as the source of molecular oxygen (Scheme VII). The product obtained was found to be a mixture of the labeled (70%) and unlabeled (30%) quinone anils as determined by analysis of the mass spectra.

Discussion

The results reported herein give direct evidence for the catalytic activity of tyrosinase on arylamines and *o*-aminophenols. These two classes of substances involve normal ca-

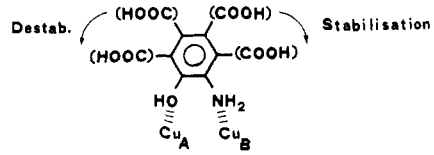


FIGURE 2: Influence of substitution on the stability of the tyrosinase/*o*-aminophenol complex (see Table I for the different values for the different isomers).

talysis, and the resulting products exhibit the expected structures for the usual hydroxylation and oxidation reactions carried out in the presence of tyrosinase.

Michaelis Constants. The K_m values obtained either for *o*-aminophenols (Table I) or for aromatic amines (Table II) indicate that no significant difference appears between these substances and the classical substrates (*o*-diphenols and monophenols). This is a consequence of the similar structural and electronic features of both interacting groups (amino and hydroxy).

From the K_m values of the four isomeric *o*-aminophenols (Table I), it is apparent that the position of the carboxyl group on the aromatic ring strongly influences the stability of the enzyme/substrate complex. As shown in Figure 2, the presence of the carboxyl group next to the amino or the hydroxy group leads to a stabilization or a destabilization of the enzyme/substrate complex, respectively.

This effect could originate from the substrate itself by different interactions between the carboxyl group and its ortho substituent. On the other hand, this effect could also be due to an asymmetry of the binuclear copper site. In this case, two configurations for the binding of an *o*-aminophenol to the two copper ions (Cu_A and Cu_B) are possible, with one displayed in Figure 2. As long as no three-dimensional structure of tyrosinase is known, we cannot decide whether the differences observed for the K_m values originate from purely electrostatic interactions or from steric hindrance.

Reaction Rates. As seen in Table II, the substitution of the hydroxy group (of a monophenol) by an amino group, to form the parent aromatic amine, is responsible for an important decrease in the reaction rates. Arylamines are converted to *o*-quinone imines from 700 to 2000 times slower than the corresponding monophenols (leading to the formation of *o*-quinones).

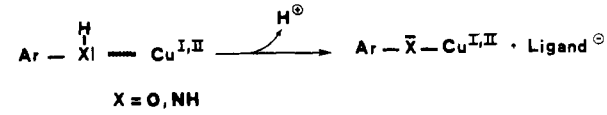
As this enzymatic conversion is a two-step reaction (Schemes I and IV), the decrease in the rates, observed with arylamines, might originate from either the first step (hydroxylation), the second one (oxidation), or both.

The results summarized in Table I, with the exception of 3,4-dihydroxybenzoic acid, clearly establish that the second step (oxidation) is hardly affected by the presence of an amino group (compare 2,3-dihydroxy with 2-amino-3-hydroxy- or 3-amino-2-hydroxybenzoic acids, for example, and 3,4-diamino with 3-amino-4-hydroxy- or 4-amino-3-hydroxybenzoic acids). This is not surprising in view of the fact that aromatic hydroxy and amino groups are oxidized at about the same electric potentials (Fieser, 1930; Suatoni et al., 1961), for identical pH and temperature conditions.

Thus, the data summarized in Tables I and II strongly suggest that the first step (hydroxylation) is responsible for the slower rates obtained when tyrosinase is incubated with aromatic amines instead of monophenols.

Most of the mechanisms proposed to describe the tyrosinase activity on monophenols (Capdevielle & Maumy, 1982; Wilcox et al., 1985) involve the formation of a covalent bond between the substrate and one of the two copper ions. This means, among others, that a deprotonation step of the hydroxy group

Scheme VIII



is involved during the reaction (Scheme VIII).

If we assume a similar pathway for arylamines, a comparison of the pK_A values of both classes of substrates should be of interest. Indeed, a large difference is found between the pK_A values of *p*-cresol, 10.26 (Barlin & Perrin, 1966), and of *p*-toluidine, 27 (McEven, 1936), which could explain the sharp decrease in the reaction rates of the arylamines whatever the reacting form of these substrates (free or complexed to the copper ions). In this context, it is interesting to note that horseradish peroxidase (EC 1.11.1.7) was found to oxidize monophenols only 10 times faster than the corresponding anilines via a radical pathway for which no prior deprotonation is needed (Job & Dunford, 1976).

Thus, the much larger difference between the rates for tyrosinase [k_{cat}(phenols)/k_{cat}(anilines) $\sim$1000] and the physicochemical data found in the literature (similar redox potentials, very different pK_A values) seem to support our interpretation of the slower reaction of arylamines with tyrosinase.

As was pointed out before, 3,4-dihydroxybenzoic acid is oxidized much faster than the related compounds listed in Table I. This compound is structurally very close to L-Dopa (3,4-dihydroxyphenylalanine), which is known to be one of the fastest reacting substrates of tyrosinase. Table I shows that a single modification of this basic structure [3,4-$(OH)_2$] either in the position [2,3-$(OH)_2$] or in the chemical nature of the substituents (3-NH_2-4-OH or 3-OH-4-NH_2) results in an important decrease of the reaction rate. However, the rates are not further affected by a second modification [2-NH_2-3-OH, 2-OH-3-NH_2, or 3,4-$(NH_2)_2$].

Product Analysis. The two reactions described in this paper clearly establish the chemical nature of the enzymatic conversions of aromatic amines and *o*-aminophenols. In both cases, *o*-quinone imines are formed in strict analogy to the classical reactions of tyrosinase with monophenols and *o*-diphenols. The *o*-quinone imines, after a nucleophilic attack of the substrate itself, were isolated as a phenoxazone (Scheme V) or a quinone anil (Scheme VI). The latter compounds have been known for a long time to be the result of an intermediate *o*-quinone imine.

The labeling experiment carried out in the presence of $^{18}O_2$ further supports that tyrosinase exhibits the same chemical reactivities toward aromatic amines and monophenols. The presence of the [4-^{16}O]quinone anil (unlabeled product) is not surprising as quinoid compounds are known to undergo in aqueous solution an exchange with water in position 4 (Teuber & Dietz, 1977; Grünanger, 1979); see Scheme VII. This exchange, at room temperature, can even be quantitative (Teuber & Dietz, 1977). Under our experimental conditions, the precipitation of the product reduced this exchange and allowed the isolation of the [4-^{18}O]quinone anil in good yield (70%).

The results reported herein clearly demonstrate the catalytic activity of tyrosinase on arylamines and provide new insights into the reactions of this enzyme. The fact that arylamines and monophenols undergo the same sequence of reactions (ortho hydroxylation, oxidation) suggests that, in both cases, the same mechanism could be involved. Finally, it should be pointed out that the hydroxylation of arylamines is not restricted to a particular source of tyrosinase, because we ob-

served the same reactions with the enzyme from the procaryote *Streptomyces glaucescens*.

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Ribonuclease Structure and Catalysis: Effects of Crystalline Enzyme, Alcohol Cryosolvents, Low Temperatures, and Product Inhibition†

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ABSTRACT: In order to determine the necessary conditions to stabilize intermediates in ribonuclease A catalysis at subzero temperatures for structural studies, we have examined the suitability of alcohol-based cryosolvents. On the basis of thermal denaturation transition curves, the enzyme is in the native conformation in high concentrations of ethanol and methanol, provided the temperature is suitably low. The effects of methanol on the catalytic properties for the hydrolysis for mono- and dinucleotide substrates also are consistent with the absence of adverse effects of the cosolvent. Significant methanolysis occurs in the presence of methanol as cosolvent. The kinetics of 2′,3′-CMP hydrolysis are complicated by severe competitive product inhibition, both in aqueous and in methanolic solvents, accounting for the previously observed effect of substrate concentration on the observed K_m. Computer-aided analysis allowed the determination of the inhibition constant as a function of experimental parameters. The reaction of ribonuclease A with 2′,3′-CMP was investigated at subzero temperatures. The turnover reaction could be made negligible at temperatures below −60 °C at pH* 3–6 in 70% methanol and below −35 °C at pH* 2.1. The rate of the catalytic reaction with crystalline enzyme was compared to that of enzyme in solution for both 2′,3′-CMP and the dinucleotide CpC. The rates were 50- and 200-fold slower, respectively, in the crystal. These investigations allowed calculation of the necessary conditions for NMR and X-ray diffraction experiments on the trapped enzyme–substrate intermediate.

The general outline of the catalytic mechanism of ribonuclease has been known for some time (Richards & Wyckoff, 1971; Usher et al., 1972; Eckstein et al., 1972). The main aspects in the hydrolysis of a polynucleotide include a two-stage process, in which the first stage involves an intramolecular displacement by the 2′-OH group of ribose of the 5′-(hydroxymethyl) group of the adjacent nucleotide to give a cyclic 2′,3′-phosphate. The second stage involves the hydrolysis of

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