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Hydroxylation of *p*-substituted phenols by tyrosinase: Further insight into the mechanism of tyrosinase activity

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ABSTRACT

A study of the monophenolase activity of tyrosinase by measuring the steady state rate with a group of *p*-substituted monophenols provides the following kinetic information: k^m_{cat} and the Michaelis constant, K^m_M. Analysis of these data taking into account chemical shifts of the carbon atom supporting the hydroxyl group (δ) and σ^+_p, enables a mechanism to be proposed for the transformation of monophenols into *o*-diphenols, in which the first step is a nucleophilic attack on the copper atom on the form E_{ox} (attack of the oxygen of the hydroxyl group of C-1 on the copper atom) followed by an electrophilic attack (attack of the hydroperoxide group on the *ortho* position with respect to the hydroxyl group of the benzene ring, electrophilic aromatic substitution with a reaction constant ρ of −1.75). These steps show the same dependency on the electronic effect of the substituent groups in C-4. Furthermore, a study of a solvent deuterium isotope effect on the oxidation of monophenols by tyrosinase points to an appreciable isotopic effect. In a proton inventory study with a series of *p*-substituted phenols, the representation of $k^{f_n}_{cat}/k^{f_0}_{cat}$ against *n* (atom fractions of deuterium), where $k^{f_n}_{cat}$ is the catalytic constant for a molar fraction of deuterium (*n*) and $k^{f_0}_{cat}$ is the corresponding kinetic parameter in a water solution, was linear for all substrates. These results indicate that only one of the proton transfer processes from the hydroxyl groups involved the catalytic cycle is responsible for the isotope effects. We suggest that this step is the proton transfer from the hydroxyl group of C-1 to the peroxide of the oxytyrosinase form (E_{ox}). After the nucleophilic attack, the incorporation of the oxygen in the benzene ring occurs by means of an electrophilic aromatic substitution mechanism in which there is no isotopic effect.

1. Introduction

Tyrosinase (E.C. 1.14.18.1) is a copper-containing enzyme widely distributed in nature. It catalyses two types of reactions: (a) the *ortho*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and (b) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Both types of reaction require molecular oxygen as the second substrate of the enzyme [1–3].

In the structure of hemocyanin [2], catechol oxidase [4] and tyrosinase of *Streptomyces castaenoglobisporus* [5], *Bacillus megaterium* [6] and *Agaricus bisporus* [7], each copper ion is coordinated by three histidines.

The first studies on aromatic ligand hydroxylation in a dinuclear Cu(I) complex by oxygen proposed that the aromatic ligand hydroxylation reaction involves an electrophilic attack on the arene ring by a (μ-η^2-η^2-peroxo)-dicopper(II) intermediate [8–10].

The first complex (μ-η^2-η^2-peroxo) described that was capable of reacting with phenolates to give catechol was described by Casella et al. [11] working with Cu(I) compounds [12]. The formation of a copper complex with O_2 was managed using anhydrous acetone at −94 °C and it was demonstrated that the oxygenated intermediate is a (μ-η^2-η^2-peroxo) dicopper (II) complex [13], as had been proposed previously [10]. It was demonstrated that when the complex reacts with lithium salts of *p*-substituted phenols, the corresponding *o*-diphenols are obtained [13]. The hyperbolic dependence of the apparent rate constant on the concentration of phenolate revealed the formation of a complex between the phenolate and the copper complex prior to the hydroxylation reaction. Using deuterated phenolates in all the carbons pointed to the absence of any isotopic effect in the hydroxylation reaction [13].

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These findings agreed with the proposal that the tyrosinase-led hydroxylation reaction must be an electrophilic aromatic substitution reaction by the side-on type peroxo intermediate [13]. Moreover, of fundamental important for this reaction to occur is that the phenol must be deprotonated, since, if this was not the case, free radicals would be produced and C–C bonds would be formed. From these observations, the authors deduced that in the active site of the enzyme the phenol must be deprotonated before undergoing hydroxylation. A similar conclusion was obtained from a study of the isotopic effect of the tyrosinase activity with deuterated phenols and *o*-diphenols [14,15]. The tyrosinase enzyme is able to catalyse these hydroxylation reactions (which do not occur via free radicals) by maintaining the distance between the copper atoms at about 3.5 Å, which is the most suitable one for accommodating the (μ-η^2-η^2-peroxo) dicopper (II) intermediate [16,17].

A kinetic and thermodynamic study was made of the reaction of the complex dicopper (I) complex [Cu_2(MeL66)] (where MeL66 is the hexadentate ligand 3,5-bis-(bis-[2-(1-methyl-1H-benzimidazol-2-yl)-ethyl]-amino]methylbenzene) with oxygen and phenolates [18]. The hydroxylation rate constant was obtained, and the representation of log *k vs.* the Hammett constants σ_p^+ pointed to a lineal relation for phenols with different substituents in C-4. The reaction constant was −1.84 [18]. Working with copper complexes with intra and intermolecular hydroxylation activities, rate constants were obtained that fitted the Hammett equation, with rate constants of −1.84 [18], −1.9 [19] and −2.2 [20]. All these values were similar to that obtained for the tyrosinase enzyme in the presence of borate and hydroxylamine (ρ = −2.4) [21], meaning that the hydroxylation in these cases also occurs through an electrophilic aromatic substitution mechanism. Working at low temperatures with high quantities of enzyme and using a poor tyrosinase substrate tyrosinase (3,5-difluorophenol), the peroxide dicopper (II) species were the major species in the monophenolase reactions [22]. The tyrosinase reaction mechanism is complex [1] and, in an attempt to simplify it, the monophenolase activity of tyrosinase was studied in the presence of borate and hydroxylamine. The added borate formed a complex with the *o*-diphenol formed, preventing the diphenolase activity. In addition, the hydroxylamine brought about the transformation of the met form to the desoxy form, an essential step for completing the catalytic cycle [21]. The apparent rate constant values obtained for the reaction of the enzyme with *p*-substituted phenols were analysed according to the Hammett equation, and the representation of log *k* vs. σ_p^+ fitted a straight line with a reaction constant, ρ, of −2,4, a similar value to those obtained for the copper complexes [18–20].

From our kinetic studies of the steady state rates of the diphenolase and monophenolase activities of mushroom tyrosinase showed that the monophenolase pathway is limiting [23–25]. The aim of this work is to obtain the rate constants for the action of tyrosinase on *p*-substituted monophenols, and to analyse them according to Hammett's relation, to obtain information on the hydroxylation mechanism, working with the enzyme in more physiological conditions (pH 7.0) and without using borate and hydroxylamine. In addition, we discuss the data for the deuterium isotope effect on the oxidation of monophenols by means of a proton inventory study to provide more information on the hydroxylation mechanism and its limiting step [13,16,22–25].

2. Materials and methods

2.1. Materials

Mushroom tyrosinase or polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1) (8300 units/mg) 3-methyl benzothiazolin-2-one hydrazone hydrochloride (MBTH) and the all substrates (phenol, 4-methylphenol, 4-hydroxyanisole, 4-*tert*-butylphenol, 4-ethoxyphenol, 4-chlorophenol, 4-bromophenol, 4-iodophenol, 4-fluorophenol and 4-hydroxybenzaldehyde) were supplied by Sigma (Madrid, Spain). All other chemicals were of analytical grade. Tyrosinase was purified according to Ref. [25]. The protein concentration was determined by Bradford's method [26], using bovine serum albumin as the standard. Stock solutions of the phenolic substrates were prepared in 0.15 mM phosphoric acid to prevent auto-oxidation. Milli-Q system (Millipore Corp.) ultrapure water was used throughout.

2.2. Spectrophotometric assays

These assays were carried out with a Perkin-Elmer Lambda-35 (Perkin-Elmer, Massachusetts, USA) spectrophotometer, on line interfaced to a PC-computer, where the kinetic data were recorded, stored and later analysed [27].

2.3. Oxymetric assays

In the case of substrates whose *o*-quinones are instable and did not form adducts with MBTH stoichiometrically, oxymetric assays were carried out [28].

2.4. Data analysis

For tyrosinase, the initial rate values (V_0) were calculated from triplicate measurements at each reducing substrate concentration, and V_0 *vs.* $[M]_0$ data were adjusted to the Michaelis–Menten equation [Eq. (1)] through the Sigma Plot 9.0 program for Windows [29].

2.5. Determination of ^{13}C NMR chemical shifts

The carbon chemical shifts given in Tables 1 and 2 were obtained from the corresponding ^{13}C NMR spectra, which were recorded at 298 K on a Bruker Avance 400 MHz instrument employing buffered solutions (at pH 7.0) of pure samples in D_2O.

3. Results and discussion

When tyrosinase acts on monophenols (see Scheme 1), for the system to reach the steady state, a low concentration of *o*-diphenol must be accumulated in the reaction medium [24]. The reaction occurs with a lag period that lasts until this level of *o*-diphenol is accumulated. Experimentally, it can be reduced by adding catalytic quantities of *o*-diphenol [30,31].

3.1. Kinetic characterisation of the monophenols studied

Based on the measurements of the steady state (V_0), by fitting a non-linear regression to Eq. (1) and taking into consideration Eq. (2), $k_{cat}^{app(m)}$ (apparent catalytic constant) and K_M^m (Michaelis constant) were calculated (Table 1). From the relation between these constants, described in Supplementary material [Eqs. (5SM, 7SM, 8SM and 10SM)], the kinetic constants k_{cat}^m shown in Table 1 were obtained.

$$V_0 = \frac{V_{max}^m [M]_0}{K_M^m + [M]_0} \quad (1)$$

where

$$V_{max}^m = k_{cat}^{app} [E]_0 \quad (2)$$

$k_{cat}^{m(app)}$ is related with k_{cat}^m through Eqs. (5SM, 7SM, 8SM and 10SM) of Supplementary material. Moreover:

$$k_{cat}^m = \frac{k_{5_1} k_{5_2}}{k_{5_1} + k_{5_2}} \quad (3)$$

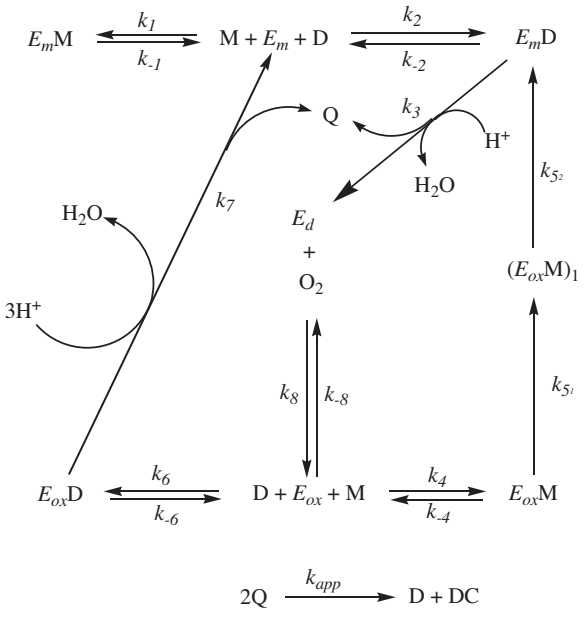
Table 1
Kinetic constants characterising the action of tyrosinase on the monophenols studied and the values of σ_p^+ and δ_1.

Phenol	k_{cat}^m (s^{-1})	K_M^m (mM)	σ_p^+	δ_1 (ppm)	R^*
Phenol	14.36 ± 1.56	0.61 ± 0.05	0	158.15	0.005
4-Methylphenol	14.76 ± 1.89	0.38 ± 0.03	−0.310	155.50	0.003
4−Hydroxyanisole	274.87 ± 12.02	0.08 ± 0.01	−0.780	152.29	None
4-*Tert*-butylphenol	5.61 ± 0.31	0.45 ± 0.03	−0.250	156.10	0.051
4-Ethoxyphenol	202.16 ± 9.87	0.17 ± 0.02	−0.810	152.39	None
4-Chlorophenol	4.60 ± 0.21	1.87 ± 0.15	0.112	156.60	0.096
4-Bromophenol	6.90 ± 0.38	1.98 ± 0.21	0.148	157.51	0.030
4-Iodophenol	2.73 ± 0.12	2.02 ± 0.21	0.132	157.42	0.030
4-Fluorophenol	10.54 ± 0.87	0.41 ± 0.04	−0.072	154.10	0.050
4-Hydroxybenzaldehyde	0.19 ± 0.01	1.14 ± 0.12	0.730	164.21	0.100

* Is the ratio $[D]_0/[M]_0$.

Table 2
Proton inventory studies for the oxidation of *p*-substituted phenols by tyrosinase and selected ^{13}C NMR chemical shifts.

Monophenol	δ_1 (ppm)	Overall isotope effect (k_H/k_D)
4-Hydroxyanisole	152.29	1.14 ± 0.1
4-Ethoxyphenol	152.39	1.43 ± 0.1
4-Hydroxybenzylalcohol	156.63	1.41 ± 0.1
4-Hydroxyphenylacetic acid	156.49	2.00 ± 0.2
4-Hydroxyphenylpropionic acid	156.13	2.17 ± 0.2
Tyramine	157.28	2.94 ± 0.3
L-Tyrosine	158.86	3.45 ± 0.3



Scheme 1. Kinetic mechanism to explain the action of tyrosinase on monophenols. E_m = mettyrosinase, E_d = deoxytyrosinase, E_{ox} = oxytyrosinase, D = *o*-diphenol, M = monophenol, Q = *o*-quinone and DC = dopachrome.

and

$$K_M^m = \frac{k_{-4}}{k_4} \tag{4}$$

As an example, the oxymetric recordings of oxygen consumption, $V_0^{O_2}$, in the case of 4-chlorophenol are shown in Fig. 1, which also shows the representation of $V_0^{O_2}$ *vs.* $[M]_0$. Analysis of these data by non-linear regression to Eq. (1) gives V_{max}^m and K_M^m. Subsequently, applying Eqs. (2) and (10SM) of the Supplementary material gives k_{cat}^m (Table 1).

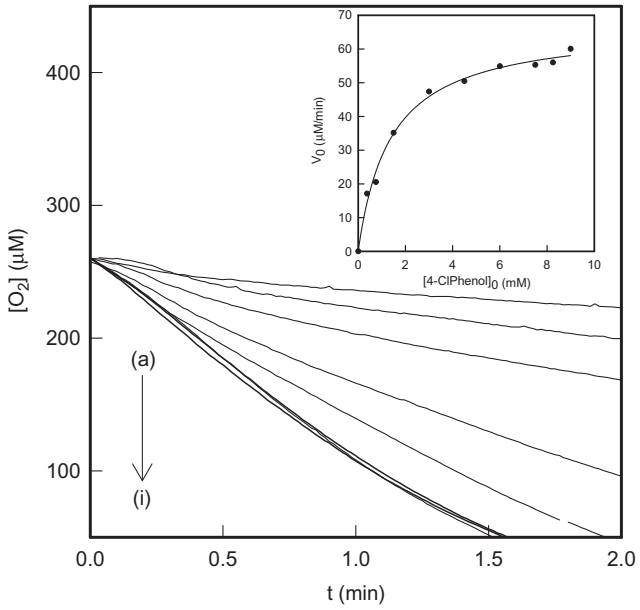


Fig. 1. Oxymetric recordings of the oxygen consumption measured in the reaction of tyrosinase on 4-chlorophenol. The experimental conditions were: 30 mM sodium phosphate buffer (pH 7.0) at 25 °C and the substrate concentrations (mM) were: 0.37 (a), 0.75 (b), 1.5 (c), 3 (d), 4.5 (e), 6 (f), 7.5 (g). 8.25 and 9 (i). 4-chlorocatechol was added to maintain the ratio [4-chlorocatechol]/[4-chlorophenol] = 0.096. Oxygen concentration: 0.26 mM and $[E]_0$ = 0.9 μM. Inset. Representation of V_0 *vs.* [4-chlorophenol].

3.2. Fitting the values of log k_{cat}^m to Hammett's equation

In a study of the hydroxylation capacity of the copper compounds acting on phenolates (with a rate constant, *k*), the values of log *k* fitted to those of σ_p^+ gave similar reaction constants (ρ), regardless of the complex used [18–20].

In addition, from the kinetic studies of the action of tyrosinase on *p*-substituted phenols in a medium containing 0.5 M borate and 6.8 mM hydroxylamine at pH 9.5, the initial rate values can be obtained. Analysis of these gives the catalytic constants of different molecules, which, when analysed by Hammett's equation, give a value of $\rho = -2.4$ [21], which is similar to those obtained using complexes of copper [18–20].

The self-assembly of a fully functional tyrosinase-like μ-η²-η²-peroxo dicopper (II) core, supported exclusively by monodentate imidazole ligands, has recently been demonstrated at extreme solution temperatures (−125 °C) [32] and it was proposed that the proteic structure does not necessarily improve the active site to carry out its catalytic activity [32]. Study of the hydroxylation rate constant of different phenolates according to Hammett's equa-

tion provided a value −2.2 for the reaction, a value that is consistent with the electrophilic aromatic substitution mechanism of hydroxylation proposed for tyrosinase (ρ =−2.4) [21] and other functional model complexes (from −1.8 to −2.4) [18–20].

In this work, we study the action of tyrosinase on a group of *p*-substituted phenols at physiological pH, which distinguishes the study from others carried out in the presence of borate or hydroxylamine in the reaction medium. Fitting log k^m_{cat} *vs.* σ_p^+ (Fig. 2) to Hammett's equation gives a value of $\rho = -1.75 \pm 0.16$ [21].

3.3. Fitting log K^m_M to Hammett's equation

For the different *p*-substituted phenols studied (Table 1), the dependences of log K^m_M with respect to σ_p^+, according to Hammett's equation, are shown in Fig. 2 Inset. Note that the dependence of k^m_{cat} and K^m_M are directly opposite. In the case of k^m_{cat}, as the donating character of the substituent in C-4 increases, so the nucleophilia of the oxygen of the hydroxyl group increases (higher k_{5_1}), and the electrophilic attack of the hydroperoxide group also increases (higher k_{5_2}) Eq. (3). However, in the case of K^m_M, since equilibrium is rapidly reached in the hydroxylation pathway, the Michaelis constant is given by Eq. (4) and the presence of an electron withdrawing group in C-4 reduces the electron density of the aromatic ring, diminishing the binding constant, k_4, possibly due to a diminution of the π–π interactions with an aromatic ring of the enzyme active site. As a result, K^m_M increases, Eq. (4), and log K^m_M *vs.* σ_p^+ (Fig. 2 Inset) provides a positive slope. This dependence of K^m_M shows that, besides electronic effects, hydrophobic and steric effects also must be taken into account in the substrate binding.

It should be noted that if studies are carried out with copper complexes the phenols to be studied by means of an intramolecular reaction should be in phenolate form so that the hydroxylation reaction can occur. In this situation, the preliminary electophilic aromatic substitution reaction, deprotonation of the hydroxyl group not take place and so is not discussed. However, in the mechanism described in Schemes 1 and 1SM (Supplementary material), when the enzyme attacks a monophenols, it is protonated. The oxygen of the hydroxyl group of the enzyme makes a nucleophilic attack on the copper atom in such a way that the catalytic constants [Eq. (3)] order themselves with the chemical shift values of the C-1 (δ_1), so that the lower the value of δ, the higher the charge density of the carbon and the more pronounced nucleophilic character of the oxygen [22]. For the electrophilic attack to occur the proton of the hydroxyl group must previously have been transferred. In the case of the form E_{ox}, it has been proposed that the proton might be transferred to the peroxide, giving rise to a hydroperoxide [33–35], which would carry out the electrophilic attack, governed by k_{5_2}, releasing a proton from the aromatic ring. Since protons participate in these reactions, the possible isotopic effects were studied and interpreted.

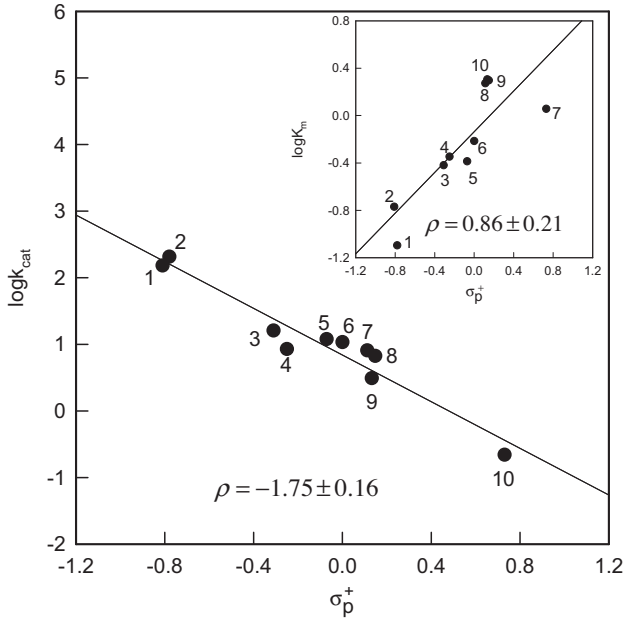


Fig. 2. Representation of log k^m_{cat} *vs.* σ_p^+ (data taken from Table 1). (1) 4-Ethoxyphenol, (2) 4-hydroxyanisole, (3) 4-methylphenol, (4) 4-*tert*-butylphenol, (5) 4-fluorophenol, (6) phenol, (7) 4-chlorophenol, (8) 4-bromophenol, (9) 4-iodophenol and (10) 4-hydroxybenzaldehyde. Inset. Representation of log K^m_M *vs.* σ_p^+ (data taken from Table 1). (1) 4-Hydroxyanisole, (2) 4-ethoxyphenol, (3) 4-methylphenol, (4) 4-*tert*-butylphenol, (5) 4-fluorophenol, (6) phenol, (7) 4-hydroxybenzaldehyde, (8) 4-chlorophenol, (9) 4-bromophenol and (10) 4-iodophenol.

3.4. Studies of the isotopic effect

As mentioned above, in the enzymatic study of the tyrosinase with monophenols, protons participate in the nucleophilic attack and in the electrophilic aromatic substitution steps.

Working with deuterated phenols in all the carbon atoms and copper complexes and tyrosinase, no isotopic effect was observed [16,19]. Therefore, the proton released during electrophilic aromatic substitution does not participate in the limiting step. However, when tyrosinase acts on deuterated monophenols in the hydroxyl group a noticeable isotopic effect was observed (Table 2), an effect that became stronger as the charge density of the carbon that supports the hydroxyl group was less (higher δ) (Table 2). Therefore, the isotopic effect demonstrated with the enzyme corresponds to the nucleophilic attack step governed by k_{5_1}. Fig. 3 shows the linear dependence of $k^{f_n}_{cat}/k^{f_0}_{cat}$ (catalytic constant with a fraction of deuterium in the solution *n* compared with the catalytic constant in water) on *n* (fraction of deuterium in the solution), confirming that only this proton transfer process is the responsible of the control of overall rate of the hydroxylation tyrosinase activity [15].

3.5. Proposed mechanism for the hydroxylation of monophenols

In the enzyme, two critical values of pK_a exist (see Scheme 1SM), as was seen when working with 3,6-difluorocatechol [35], and with deoxy-tyrosinase [36]. This might be explained by the existence of two bases, B_1 and B_2, that participate in the catalytic cycle (see Scheme 1SM). The met-tyrosinase form may bind to both monophenols and *o*-diphenols, by accepting base B_1, a proton. This form is inactive on monophenol (M) and active on *o*-diphenol (D). The met-tyrosinase oxidises D to *o*-quinone (Q) and E_m passes to E_d, which, with oxygen, generates E_{ox}, which carries out the hydroxylation of the monophenol.

The binding of E_{ox} to M may involve the transfer of the proton of the hydroxyl group to the peroxide since base B1 is protonated, generating [33–35] a hydroperoxide, which may carry out the hydroxylation through an electrophilic aromatic substitution. The carbon atom on which the hydroxylation takes place loses a proton, which may be transferred to base B2 [35,37,38]. After this hydroxylation reaction, the *o*-diphenol generated cannot be oxidised because the orbitals of the oxygen atoms are not co-planar with the atoms of copper. In such a situation, the *o*-diphenol generated must be released from an copper atom and bind properly again (coplanar) or be released definitively to the medium. Confirmation of the release of the *o*-diphenol by mass spectroscopy lends weight to the proposed mechanism [39].

In summary, the kinetic data provided in this study support the mechanism proposed for the action of copper complexes on phenolates [18–20] and for the action of the enzyme on *p*-substituted

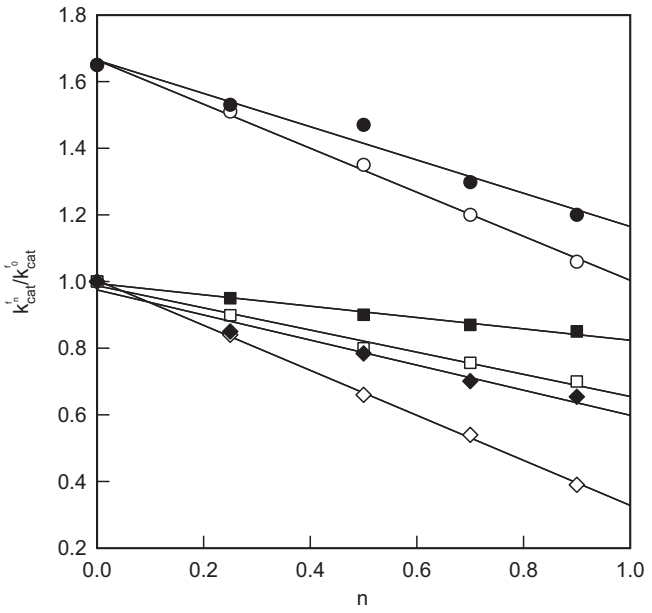


Fig. 3. Plots of $k^{f_n}_{cat}/k^{f_0}_{cat}$ ratio against n (atomic fraction of deuterium) for monophenols: (●) 4-hydroxyphenylacetic acid, (○) 4-hydroxyphenylpropionic acid, (◇) tyramine, (■) 4-hydroxyanisole, (□) 4-ethoxyphenol, (◆) 4-hydroxybenzylalcohol. The experimental conditions were 50 mM sodium phosphate buffer (pH 7.0) at 25 °C, 2% dimetylformamide, 2 mM MBTH for tyramine, 2.5 mM for 4-hydroxyanisole, 4-ethoxyphenol and 4-hydroxybenzylalcohol and 4 mM for 4-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid and different concentrations of substrate. The enzyme concentrations were 4.92 nM for 4-hydroxyanisole, 24.6 for 4-ethoxyphenol and 4-hydroxybenzylalcohol and 49.2 nM for tyramine, 4-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid (data are taken from [15]).

phenols [21]. However, it should be noted that the experimental data were obtained at physiological pH and in the absence of borate and hydroxylamine. It should be added that the study of the isotopic effect working with deuterated phenols in the hydroxyl group showed that the nucleophilic attack (governed by k_{5_1}) may be slower than the electrophilic attack (governed k_{5_2}), since the dependence with regard to Hammett's equation is the same for both steps. These experimental data obtained in steady state conditions support the mechanism proposed in Scheme 1SM (Supplementary material).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.074.

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