Regioselective Oxidation of Phenols Catalyzed by Polyphenol Oxidase in Chloroform

Rafi Z. Kazandjian and Alexander M. Klibanov*

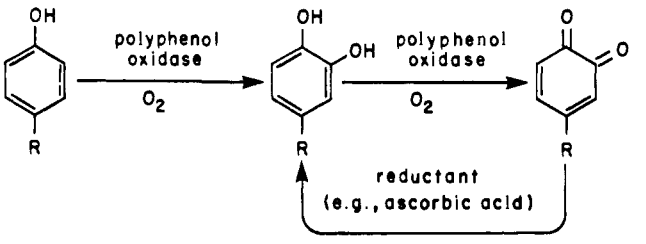
Contribution from the Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received February 11, 1985*

Abstract: Mushroom polyphenol oxidase can catalyze the oxidation of a number of phenols to *o*-quinones in chloroform. While in water the enzymatic oxidation results in negligible yields due to rapid inactivation of the enzyme and spontaneous polymerization of the quinones, in chloroform a quantitative conversion has been achieved; the quinones produced have been nonenzymatically reduced to catechols, thereby affording a net regioselective polyphenol oxidase catalyzed hydroxylation of phenols.

The enzyme polyphenol oxidase catalyzes hydroxylation of phenols with O_2 to catechols and subsequent dehydrogenation to *o*-quinones.[2,3] Since regioselective oxidation of aromatic compounds is often desirable but is a difficult problem in organic chemistry,[4] the above enzymatic reactions appear to be of great preparative interest. However, attempts to use polyphenol oxidase as a practical catalyst have been unsuccessful because the instability of *o*-quinones in water[5] causes rapid polymerization to polyaromatic pigments,[6] also inactivating the enzyme.[3,7]

Inspection of the mechanism of *o*-quinone polymerization[8] reveals that water is an essential component of that reaction. This seems to rule out polyphenol oxidase as a useful catalyst because enzymatic processes take place in aqueous solutions. However, in the present work we have discovered that polyphenol oxidase can vigorously function in chloroform whereby a number of phenols can be quantitatively converted to stable *o*-quinones, which can then be nonenzymatically reduced to catechols, thus affording an efficient and regioselective net hydroxylation of phenols (Scheme I).

Scheme I



Results and Discussion

A powdered commercial preparation of mushroom polyphenol oxidase was added to a solution of *p*-cresol in chloroform, and the suspension[9] was vigorously shaken under air. Periodically, aliquots were withdrawn and their absorption spectra measured. No significant changes were detected, indicating that no reaction occurred. However, when the same experiment was performed with an enzyme sample that had been dissolved in an aqueous buffer at pH 7 (optimum pH of the enzymatic activity), followed by its precipitation with acetone and drying, a significant increase in absorbance with time was observed (Figure 1, curve b). This marked acceleration of the reaction caused by the pretreatment of polyphenol oxidase can be attributed to the fact that the enzyme "remembers" the pH of the last aqueous solution to which it has been exposed (i.e., the enzyme's ionogenic groups acquire the corresponding ionization states that then remain both in the solid state and in chloroform).[10]

When the time course of the oxidation of *p*-cresol catalyzed by the "pH-optimized" polyphenol oxidase in chloroform was followed by gas chromatography, no appreciable decrease in the substrate concentration was detected. This means that although the reaction takes place, it is very slow (less than 5% degree of conversion after 3 h). Since the enzyme in chloroform is present in the form of a suspension,[9] it was likely that the reaction, as in many other heterogeneous catalytic processes,[11] was limited by diffusion. To lower/eliminate intraparticle diffusional limitations, we precipitated the enzyme from pH 7 onto glass powder (this results in a thin layer of the biocatalyst on the glass surface and consequently in a more efficient utilization of the available enzyme than in its suspension). Such immobilization enhanced the rate of the enzymatic oxidation by a factor of 6. The rate of the reaction increased even more when 0.5% of the aqueous buffer (pH 7) was added to the chloroform solution beforehand[12] (Figure 1, curve d). GC analysis showed that after 3 h of reaction catalyzed by immobilized and hydrated polyphenol oxidase, there was no *p*-cresol left. HPLC analysis confirmed the disappearance of the substrate and appearance of a new peak. When the reaction product was reduced with an aqueous solution of ascorbic acid

(1) This enzyme (EC 1.14.18.1) is also known as tyrosinase, catecholase, cresolase, phenolase, and catechol oxidase.

(2) Mason, H. S. *Adv. Enzymol. Relat. Subj. Biochem.* **1957**, *19*, 79-233. Malmstrom, B. G.; Ryden, L. In "Biological Oxidations"; Singer, T. P., Ed.; Interscience: New York, 1968; pp 419-428.

(3) Vanneste, W. H.; Zuberbuhler, A. In "Molecular Mechanisms of Oxygen Activation"; Hayaishi, O., Ed.; Academic Press: New York, 1974; pp 371-404.

(4) Gunstone, F. D. *Adv. Org. Chem.* **1960**, *1*, 103-147. Norman, R. O. C.; Taylor, R. "Electrophilic Substitution in Benzenoid Compounds"; Elsevier: Amsterdam, 1965; Chapters 5 and 12. Stoddart, J. F. "Comprehensive Organic Chemistry"; Pergamon Press: Oxford, 1979; Vol. 1.

(5) E.g., for polyphenol oxidase catalyzed conversion of L-tyrosine to L-DOPA, see: Wykes, J. R.; Dunnill, P.; Lilly, M. D. *Nature (London)* **1971**, *230*, 187. Sih, C. J.; Foss, P.; Rosazza, J.; Lemberger, M. *J. Am. Chem. Soc.* **1969**, *91*, 6204. Vilanova, E.; Manjon, A.; Iborra, J. L. *Biotechnol. Bioeng.* **1984**, *26*, 1306-1312. In these studies measurable concentrations of L-DOPA could be obtained only when a large molar excess of a reductant (ascorbic acid) was present during the reaction to reduce the quinone formed to the catechol form.

(6) This process is responsible for the well-known phenomenon of browning of fruits and vegetables (Whitaker, J. R. "Principles of Enzymology for the Food Sciences"; Marcel Dekker: New York, 1972; Chapter 22) and can also be used for the enzymatic removal of phenols from industrial wastewaters (Atlow, S. C.; Bonadonna-Aparo, L.; Klibanov, A. M. *Biotechnol. Bioeng.* **1984**, *26*, 599-603).

(7) Wood, B. J. B.; Ingraham, L. L. *Nature (London)* **1965**, *205*, 291. Dietler, C.; Lerch, K. In "Oxidases and Related Redox Systems"; King, T. E., Mason, H. S., Morrison, M., Eds.; Pergamon Press: Oxford, 1982; pp 305-317.

(8) Dawson, C. R.; Tarpley, W. B. *Ann. N.Y. Acad. Sci.* **1963**, *100*, 937-948.

(9) The enzyme is insoluble in chloroform.

(10) The same phenomenon was observed with porcine pancreatic lipase: Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192-3196. It is worth noting that when polyphenol oxidase was precipitated from a *nonbuffered* aqueous salt solution, the enzyme exhibited no oxidative activity (Figure 1, curve c), thus signifying the role of the pH.

(11) Smith, J. M. "Chemical Engineering Kinetics", 3rd ed. McGraw-Hill: New York, 1981; Chapter 11.

(12) We have examined the dependence of the enzymatic reaction rate in this system on the amount of added aqueous buffer: the rate greatly increased when water content exceeded 0.1% (approximately the solubility of water in chloroform) and then leveled off at the water content of 0.5%, which evidently secures the necessary degree of hydration.

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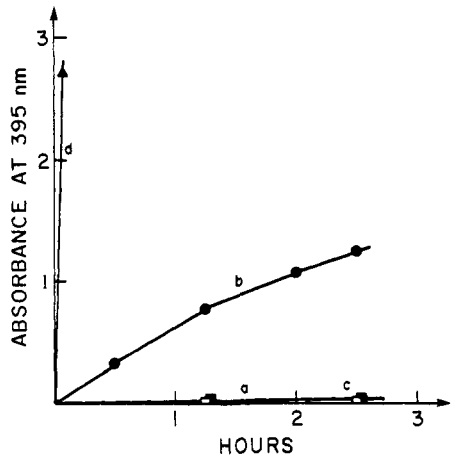

Figure 1. Time course of the polyphenol oxidase catalyzed oxidation of *p*-cresol to 4-methyl-1,2-benzoquinone in chloroform. (a) Commercial preparation of the enzyme. (b) The enzyme precipitated from an aqueous solution at pH 7 (50 mM phosphate buffer). (c) Same as (b) but for a nonbuffered aqueous solution (0.1 M NaCl). (d) Prior to the reaction the enzyme was precipitated from the buffered aqueous solution (pH 7) onto glass powder, placed in chloroform, and then hydrated with 0.5% (v/v) (with respect to chloroform) of the aqueous buffer. Conditions: 1 mL of 50 mM *p*-cresol in chloroform, 1 mg of polyphenol oxidase (free or immobilized), 100 mg of the glass powder, shaking at 250 rpm at 25 °C under air.

prior to an HPLC run, that new peak was replaced by another, which coincided with that of authentic 4-methylcatechol. Therefore, immobilized and hydrated polyphenol oxidase *quantitatively* converted *p*-cresol to 4-methyl-1,2-benzoquinone in chloroform[13] (Scheme I, where R = CH_3).

It should be stressed that the above complete enzymatic conversion is possible in chloroform but *not* in water. When 1 mg of polyphenol oxidase is added to 1 mL of a 50 mM aqueous solution of *p*-cresol (50 mM phosphate buffer, pH 7), the quinone concentration rapidly reaches about 0.4 mM, and then the reaction stops due to total inactivation of the enzyme.[14] Even the quinone initially formed readily polymerizes in the aqueous solution.[8] Additional advantages of the enzymatic oxidation in chloroform[15] vs. water are a 10-fold higher solubility[16] of O_2 and a greater solubility of most phenols.

Two dozen other phenols have been investigated as substrates for immobilized and hydrated polyphenol oxidase in chloroform, and about half of them were reactive (R = H, CH_3, CH_3O, $HOOCCH_2CH_2$, Cl, Br, I, $HOCH_2$, $HOCH_2CH_2$, and $C_6H_5CONHCH_2$).[17] The substituent in the ring must be in the para position (e.g., *o*- and *m*-cresols are unreactive). The reactivity decreases upon a transition of R from electron-donating to electron-withdrawing substituents. Bulky phenols (*p*-phenylphenol, 5-indanol, *p-tert*-butylphenol, and 1- and 2-naphthols) were unreactive. A non-phenol, *p*-toluidine, was also oxidized enzymatically.[19]

Thus the substrate specificity of polyphenol oxidase in chloroform is similar to that in water.[2] However, in contrast to water, chloroform can be used as the reaction medium for preparative, polyphenol oxidase catalyzed transformations according to Scheme I. This was illustrated by the conversion of a tyrosine derivative (the free amino acid is insoluble in chloroform) to a derivative of L-DOPA (a drug used for the treatment of Parkinson's disease[20]). *N*-Acetyl-L-tyrosine ethyl ester (1 mmol) was dissolved in 40 mL of chloroform, and the solution was placed in a 250-mL Erlenmeyer flask. Then 40 mg of polyphenol oxidase precipitated onto 4 g of glass powder from pH 7 were added, followed by addition of 0.2 mL of 50 mM aqueous phosphate buffer (pH 7) to hydrate the enzyme. The suspension was vigorously shaken under air at 25 °C for 4 h after which time the substrate was fully depleted (determined by GC). The red (due to the quinone) chloroform solution was separated from the enzyme by decantation and then extracted with an equal volume of 100 mM ascorbic acid in water. The aqueous extract was evaporated under vacuum, and the residue was extracted with ethyl acetate and purified on a silica gel column, followed by evaporation of the solvent. The product (0.71 mmol, 71% of the theoretical yield) was homogeneous by TLC and HPLC and was positively identified as *N*-acetyl-3,4-dihydroxyphenylalanine ethyl ester by (i) colorimetric assay for catechols,[18] (ii) mass spectrometry, and (iii) comparative HPLC analysis following alkaline hydrolysis (3 N NaOH, 65 °C, 3 h under N_2) of the product to DOPA.

In conclusion, mushroom polyphenol oxidase has been found to be catalytically active in chloroform. While the enzymatic oxidation of phenols in water is preparatively useless due to negligible yields, rapid inactivation of the enzyme, and spontaneous polymerization of the products, the same reaction in the organic solvent affords quantitative transformations depicted in Scheme I.

Experimental Section

Materials. Mushroom polyphenol oxidase[1] was purchased from Sigma Chemical Co. (where it is listed as tyrosinase) as a solid with a specific activity of 2430 units/mg (1 unit is defined as the enzyme activity re-

(13) The polyphenol oxidase catalyzed conversion of *p*-cresol to 4-methyl-1,2-benzoquinone in chloroform obeys Michaelis–Menten kinetics with K_m = 12.5 mM and V = 1.2 μmol/min·mg enzyme (conditions are the same as in Figure 1d).

(14) In chloroform the reaction inactivation[3,7] of polyphenol oxidase is dramatically reduced compared to water: even after complete conversion of *p*-cresol to 4-methyl-1,2-benzoquinone, a third of the enzymatic activity is retained.

(15) We examined the polyphenol oxidase catalyzed oxidation of *p*-cresol in a number of solvents other than chloroform. In all instances, the enzyme precipitated (dried) onto glass powder from pH 7 was employed, and all organic solvents contained 0.5% (v/v) of the aqueous buffer (pH 7). The enzyme was found to be capable of oxidizing *p*-cresol in many hydrophobic solvents such as methylene chloride, carbon tetrachloride, dichloroethane, benzene, toluene, hexane, butyl acetate, and diisopropyl ether. On the other hand, the enzyme was completely inactive in more hydrophilic solvents such as ether, ethyl acetate, ethanol, butanol, acetone, and acetonitrile. These results can be readily explained in terms of the following rationale: hydrophilic solvents strip the essential water from the enzyme (thereby inactivating it) by partitioning, whereas in very hydrophobic solvents, having virtually no affinity for water, the essential water remains bound to the enzyme, which thus retains its catalytic activity.

The rate of the enzymatic oxidation of *p*-cresol in the "suitable" (hydrophobic) organic solvents was lower than or comparable to that in chloroform. The only exception was hexane where the rate of the substrate disappearance was twice as fast. However, the product (4-methylcatechol) recovery in hexane, following complete polyphenol oxidase catalyzed oxidation of *p*-cresol and subsequent reduction with ascorbic acid, was only a few percent (as opposed to the nearly quantitative recovery in chloroform). No low molecular weight products were detected by GC, HPLC, or TLC, suggesting that in hexane enzymatically formed 4-methyl-1,2-benzoquinone rapidly polymerizes even in the presence of just 0.5% water.

(16) Stephen, H., Stephen, T., Eds. "Solubilities of Inorganic and Organic Compounds"; Pergamon Press: Oxford, 1963; Vol. 1 p 7.

(17) The relative rates of the enzymatic oxidation in that series of phenols (measured by GC on the basis of the substrate disappearance) were 15, 100, 40, 42 (in a 3:1 (v/v) mixture of chloroform and butyl acetate to dissolve *p*-hydroxyphenylpropionic acid), 5, 5, 5, 70 (in a 1:1 (v/v) mixture of chloroform and butyl acetate to dissolve *p*-hydroxybenzyl alcohol), 22, and 7 (both in a 9:1 (v/v) mixture of chloroform and amyl alcohol to dissolve *p*-hydroxyphenylethyl alcohol), respectively. In the case of the slower substrates, the enzyme concentration was then increased from 1 to 4 mg/mL, and as a result, nearly 100% conversion was achieved for all of the substrates after several hours (the exact time depends on the nature of the substrate) except for the *p*-halophenols, where the maximal degree of conversion was 80%. The enzymatically produced quinones were reduced with ascorbic acid to form substituted catechols identified by Arnow's procedure.[18] The only exceptions were phenol and (*p*-hydroxyphenyl)propionic acid, where the enzymatically formed quinones were unstable and rapidly polymerized.

It is worth noting that polyphenol oxidase is regioselective even if no substituent is present in the para position of the phenolic substrate: enzymatic oxidation of phenol, followed by reduction with ascorbic acid resulted in the formation of catechol only, with no hydroquinone detected (by GC or HPLC).

(18) Waite, J. H.; Tanzer, M. L. *Anal. Biochem.* **1981**, *111*, 131–136.

(19) The rate of this reaction was about 5% of that with *p*-cresol under the same conditions. The maximal degree of the enzymatic conversion of this substrate was 80%.

(20) Stern, D., Ed. "The Clinical Uses of L-DOPA"; Lankaster Medical and Technical Publishing: London, 1975.

(21) This work was financially supported by W. R. Grace & Co. R. Z. K. is a Procter & Gamble graduate fellow.

sulting in an increase in absorbance at 280 nm of 0.001/min at pH 6.5 at 25 °C in a 3-mL reaction volume containing L-tyrosine).

Glass powder (100–300 mesh) was obtained from Amend Drug & Chemical Co. (New York City) and was washed with 10% nitric acid prior to use; other varieties of glass beads have been successfully used as well.

All chemicals used in this work were obtained from commercial suppliers and were of the highest purity available.

Preparation of the Enzyme. (i) **Acetone Precipitation.** Polyphenol oxidase (20 mg) was dissolved in 6 mL of 50 mM phosphate buffer (pH 7), and the solution was cooled to 4 °C. Then 10 mL of cold (−20 °C) acetone was gradually added with vigorous stirring at 4 °C, and the mixture was left at −20 °C overnight. The precipitated solid was recovered by filtration on a Buchner funnel.

(ii) **Precipitation onto Glass Powder.** Polyphenol oxidase (6 mg) was dissolved in 0.3 mL of 50 mM phosphate buffer (pH 7), and then 600 mg of glass powder was added. The slurry was thoroughly but gently mixed, spread on a watch glass, and left to dry at room temperature with occasional mixing until visibly dry (freely flowing) beads were obtained. Alternatively, the enzyme could be precipitated onto glass powder with acetone by the procedure outlined in (i). The two methods resulted in similar activities of the precipitated polyphenol oxidase in chloroform. Since drying is much faster than acetone precipitation (2.5 vs. about 18 h) and also more convenient, it was used throughout this study for "immobilization" of polyphenol oxidase onto glass powder.

Reduction with Ascorbic Acid. Enzymatically produced 1,2-quinones were reduced to the corresponding catechols by ascorbic acid. Following removal of the enzyme by decantation, 0.5 mL of the chloroform solution was added to 0.5 mL of a 100 mM solution of ascorbic acid in water. The mixture was vigorously shaken with a Vortex mixer for 1 min, and then the aqueous phase was analyzed as described below.

Assays. Several different methods of analysis were employed.

(i) **Spectrophotometry.** Aliquots of the chloroform solution were withdrawn and their absorption spectra measured in the range 360–600 nm. In the case of the enzymatic oxidation of *p*-cresol in chloroform, the maximum absorbance for 4-methyl-1,2-benzoquinone was at 395 nm; the extinction coefficient at that wavelength was determined to be 1.4 $mM^{-1}\cdot cm^{-1}$.

(ii) **GC.** All gas chromatographic measurements were carried out with a 5-m, 530-μm fused-silica capillary column (Hewlett-Packard) (N_2 carrier gas, 30 mL/min; detector and injector port temperature 250 °C) in an appropriate temperature regime.

(iii) **HPLC.** Following the reduction of the enzymatically formed 1,2-quinones with ascorbic acid, the aqueous solutions containing the corresponding catechols were analyzed by HPLC by using a C_{18} reverse-phase μBondapak column (Waters Associates) with an acetonitrile–10 mM phosphate buffer (pH 3) gradient elution system. The products were detected on the basis of their absorbance at 254 nm.

(iv) **Arow's Assay.** Catechols were determined by the colorimetric method.[18] The extinction coefficients at 500 nm were taken from ref 18. Under the assay conditions, monophenols and ascorbic acid do not interfere.

(v) **TLC** analysis on the product of the enzymatic oxidation of *N*-acetyl-L-tyrosine ethyl ester was carried out with silica gel plates and ethyl acetate as a solvent; R_f values were 0.35 for the substrate and 0.25 for the product (ascorbic acid did not migrate).

Stable Simple Enols. 10.[1a] 500-MHz ^{1}H NMR of Trimesitylethenol and Deuterated Analogues: Complete Signal Assignments and Direct Determination of Equilibrium Steric Isotope Effects from the NMR Integrals of the Isotopomeric Enols

Silvio E. Biali,[1b] Zvi Rappoport,*[1b] and William E. Hull[1c]

Contribution from the Department of Organic Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, and Bruker Analytische Messtechnik GmbH, D-7512 Rheinstetten 4, West Germany. *Received December 4, 1984*

Abstract: *E*/*Z* mixtures of the isotopomers Mes*C(Mes)=C(OH)Mes (**7a**/**7b**) and Mes*C(Mes)=C(OH)Mes* (**8a**/**8b**) [Mes = 2,4,6-$(CH_3)_3C_6H_2$; Mes* = 2,4,6-$(CD_3)_3C_6H_2$] were prepared by addition of MesMgBr and Mes*Br to Mes*C(Mes)=C=O. High resolution ^{1}H NMR at 500 MHz of trimesitylethenol (**5**) and the labeled isotopomers together with NOE data, saturation transfer, and T_1 relaxation times have now led to a complete assignment of the signals for all 15 nonequivalent proton sites in these triarylvinyl propellers. Two OH signals were observed for each isotopomeric pair. The relative integration of the two OH signals and of the methyl signals of the β-ring (cis to α-Mes) and the β′-ring (cis to OH) gave the isotopomeric ratio. Lorentz–Gaussian line-shape transformations, base-line corrections, and several repeated integrations at 500 MHz were used in order to obtain reliable ratios. The *E* isomers **7b** and **8b** (β-ring = Mes*) were present in excess, and the best values of the isotope effects at 302 K are 1.026 ± 0.007 for the ratio **7b**/**7a** and 1.048 ± 0.027 for **8b**/**8a**. A statistical analysis showed that the values are significantly different from unity. The isotopomer ratios were ascribed to an equilibrium steric isotope effect where the isomer with the smaller Mes* group cis to the α-mesityl ring is present in a slight excess. The appearance of a separate OH signal for each isotopomer results from slightly different conformations of the labeled derivatives due to the steric isotope effect. This is the first *direct* determination of a steric isotope effect by simple integration of NMR signals.

Steric isotope effects[2] arise from the different effective "size" of isotopic atoms. The covalent C–D bond has a mean bond length which is 0.005 Å shorter than that for C–H, and that for benzene-d_6 is about 3% smaller than that for benzene.[3] A more accurate representation is that steric constraint usually raises vibrational frequencies of hydrogen, leading to a greater zero-point energy. The replacement of hydrogen by deuterium leads to a lower zero-point energy. Hence, putting the deuterium in a constrained rather than the unconstrained situtation decreases the energy more, and the deuterium is favored in the constrained location. This is reflected in both kinetic[2,4] and equilibrium steric isotope effects. Determination of the former is exemplified by

(1) (a) Part 9: Kaftory, M.; Biali, S. E.; Rappoport, Z. *J. Am. Chem. Soc.* **1985**, *107*, 1701. (b) Hebrew University. (c) Bruker Analytische Messtechnik.

(2) Reviews: (a) Carter, R. E.; Melander, L. *Adv. Phys. Org. Chem.* **1973**, *10*, 1. (b) Melander, L.; Saunders, W. H. "Reaction Rates of Isotopic Molecules"; Wiley: Chichester, 1980; pp 189–199.

(3) Bartell, L. S.; Roskos, R. R. *J. Chem. Soc.* **1966**, *44*, 457.

(4) Melander, L.; Carter, R. E. *Acta Chem. Scand.* **1964**, *18*, 1138.

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