THE ROLE OF HYDROGEN PEROXIDE IN DIOXYGEN INDUCED HYDROXYLATION OF SALICYLIC ACID

Kamil LANG, Dana M. WAGNEROVA and Jirina BRODILOVA

Institute of Inorganic Chemistry,
Academy of Sciences of the Czech Republic, 160 00 Prague 6, The Czech Republic

Received May 26, 1994
Accepted July 25, 1994

The photochemically initiated oxidation of salicylic acid by molecular oxygen in the presence of $[Fe(C_2O_4)_3]^{3-}$ leads to a mixture of 2,3- and 2,5-dihydroxybenzoic acids. Iron(II) generated by the photoreduction is reoxidized by dioxygen. Hydrogen peroxide formed in this reaction takes part in the Fenton reaction in the presence of Fe(II). Experiments with $OH^{\bullet}$ radical scavengers document the role of the radicals in the photochemical and thermal hydroxylation of salicylic acid.

Oxidations by dioxygen involving the insertion of an oxygen atom into the molecule of the substrate (SH) proceed by a complicated mechanism, the overall reaction being[1]

$$SH + O_2 + 2\ H^+ \xrightarrow{-\ 2\ e^-} SOH + H_2O\ . \qquad (A)$$

Aromatic substrates are, as a rule, hydroxylated. The activation energy of reaction (*A*) is high but the reaction is catalyzed by transition metal complexes or by enzymes. The enzyme cytochrome P-450 (monooxygenase), which catalyzes the oxidative metabolism[1] of many chemical compounds, activates molecular oxygen forming the reactive intermediate dioxygen–ferrous heme complex–substrate $[S–Fe^{2+}–O_2]$, which is electronically equivalent to the intermediate superoxo–ferric heme complex–substrate $[S–Fe^{3+}–O_2^-]$. On the other hand, when biomimetic models of this enzyme are used[2,3], such as transition metal porphyrins, the hydroxyl radicals $OH^{\bullet}$ are among possible reactive intermediates. Nonradical oxygenation, where adducts of iron with dioxygen are the active intermediates, also proceed in nonaqueous media[4,5].

In the Udenfriend system[6], which is the first functional model of a monooxygenase, insertion of an oxygen atom into the substrate occurs, iron(II) being the catalyst and ascorbic acid (or e.g. tetrahydropteridine, alloxane) the two-electron donor (see Eq. (*A*)). The nonradical mechanism was assumed for this system; thorough kinetic studies[7], however, indicate that hydrogen peroxide and $OH^{\bullet}$ radicals take an important part in this process as well. The radical mechanism involves reduction of dioxygen to H_2O_2 by

transition metal complexes in lower oxidation state (refs[5,6]). Hydroxyl radicals (Eq. (*B*)) or iron peroxo complexes (Eq. (*C*)) then act as the hydroxylation agents[7–9].

$$L_kFe^{2+} + H_2O_2 \longrightarrow L_kFe^{3+} + OH^{\bullet} + OH^- \qquad (B)$$

$$L_kFe^{2+} + H_2O_2 \longrightarrow L_kFe(IV)=O + H_2O \qquad (C)$$

The actual nature of the hydroxylating agent in many systems has been still subject to discussions[10,11].

The present work is aimed at gaining a deeper insight into the role of the photochemical generation of Fe(II) and formation of hydrogen peroxide during hydroxylation in a system containing molecular oxygen and substrate.

EXPERIMENTAL

Salicylic acid p.a. (Reanal Budapest), phenylalanine p.a. (Reanal Budapest), $(NH_4)_2Fe(SO_4)_2 . 6 H_2O$ p.a. (Lachema, Brno), ethylenediaminetetraacetic acid disodium salt (EDTA) p.a. (Lachema, Brno), $FeCl_3$ p.a. (Cambrian Chemicals), potassium oxalate p.a. (Lachema, Brno), thiourea p.a. (Lachema, Brno), sodium formate p.a. (Lachema, Brno) fungal catalase (Calbiochem), superoxiddismutase from bovine erythrocytes (Sigma), and albumin fraction V. from bovine blood (Merck) were used as received.

The concentrations of substrate and hydroxylation products were determined by HPLC, using a HPP 4001 high-pressure pump, an LCD 2563 UV-VIS detector (both Laboratorni pristroje Praha) or a PU 4025 UV detector (Philips). The chromatographic glass column, 150 mm × 3.3 mm i.d., was packed with Separon SGX C18 7 μm (Tessek Praha). The elution mixture for the determination of salicylic acid at 207 or 313 nm and of 2,3- and 2,5-dihydroxybenzoic acids at 207 nm contained methanol, water and 1 M H_3PO_4 in the ratio of 35 : 60 : 5.

A Philips spectral lamp type 93146E (90 W) was used for irradiation unless stated otherwise. Some experiments were performed using an RVK 400 medium-pressure discharge lamp (Tesla Holesovice) as a more intense source. The entire spectral region was used for the irradiation. A quartz cell containing 50 ml of the reaction solution was accommodated in a block thermostatted at 25 °C. The basic composition was as follows: salicylic acid (0.8 mmol dm^{-3}), $FeCl_3$ (0.06 mmol dm^{-3}), $K_2C_2O_4$ (3 mmol dm^{-3}). Samples for HPLC were taken in 30 min intervals. The solution was bubbled with oxygen for stirring and ensuring a constant concentration of O_2. The intensity of light 300 – 410 nm wavelength was determined by using a tris(oxalato)ferrate(III) actinometer[12]; the values obtained were $2 . 10^{16}$ photons s^{-1} dm^{-3} for the Philips spectral lamp and $1 . 10^{18}$ photons s^{-1} dm^{-3} for the RVK 400 discharge lamp. The concentration of the photoreduced Fe(II) was determined spectrophotometrically in the form of its complex with *o*-phenanthroline.

Thermal hydroxylation of the substrates with dioxygen was performed in phosphate buffer pH 7.1; the initial concentrations of $(NH_4)_2Fe(SO_4)_2 . 6 H_2O$ and EDTA were 1.3 and 1.6 mmol dm^{-3}, respectively. The solution was saturated with air (dioxygen concentration 0.28 mmol dm^{-3}). After 20 min, when all iron(II) had been oxidized by dioxygen, the product was analyzed by HPLC.

RESULTS AND DISCUSSION

Photochemical Hydroxylation

Iron(III) oxalate used in this hydroxylation system is reduced[13] by UV light according to

$$[Fe(C_2O_4)_3]^{3-} \longrightarrow [Fe(C_2O_4)_2]^{2-} + CO_2 + CO_2^- \quad . \qquad (D)$$

The reduction rate in the O_2-saturated solution depends on the total concentration of oxalate (Figs 1, 2). The figures demonstrate that hydroxylation of salicylic acid correlates with the photoreduction of iron(III); 2,3- and 2,5-dihydroxybenzoic acids are the major products. For an initial oxalate concentration of 3 mmol dm^{-3}, nearly 100% of the initial iron(III) is reduced after 60 min of irradiation. After 150 min of irradiation, when a major part of oxalate has decomposed, the concentration of iron(II) decreases. If the initial oxalate concentration is 0.3 mmol dm^{-3}, the concentrations of iron(II) and the hydroxylated products increases slowly because the actual concentration of the photochemically active $[Fe(C_2O_4)_3]^{3-}$ (log K_3 = 20.2; ref.[14]) is low due to the competitive formation of the Fe(III) complex with salicylic acid (log K_3 = 36.8; ref.[14]) or with the hydroxylation products. When the more intense radiation source (RVK 400) is used, the concentration of each of the products is 0.107 mmol dm^{-3} after 20 min of exposure. Hydroxylated products are not formed in the absence of both oxalate and iron(III).

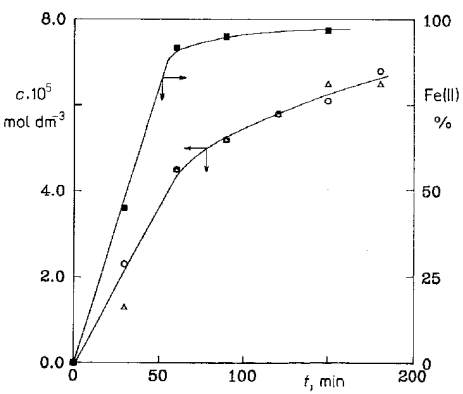

FIG. 1

Increase in concentration of Fe(II) (■), 2,5-dihydroxybenzoic acid (Δ) and 2,3-dihydroxybenzoic acid (○) during irradiation. Initial concentrations (mmol dm^{-3}): salicylic acid 0.8, potassium oxalate 3, Fe^{3+} 0.06; t = 21 °C, Hg lamp Philips 90 W

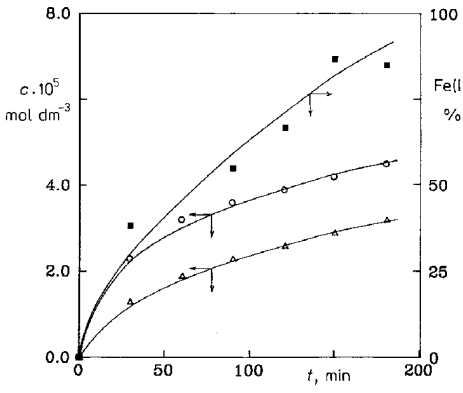

FIG. 2

Increase in concentration of Fe(II) (■), 2,5-dihydroxybenzoic acid (Δ) and 2,3-dihydroxybenzoic acid (○) during irradiation. Initial concentrations (mmol dm^{-3}): salicylic acid 0.8, potassium oxalate 0.3, Fe^{3+} 0.06; t = 21 °C, Hg lamp Philips 90 W

The hydroxylation yield in 120 min of irradiation is 70% lower in the presence of the enzyme catalase (0.1 μmol dm^{-3}), which catalyzes the decomposition of hydrogen peroxide. The spin trapping method using DMPO did not indicate any formation of the $OH^{\bullet}$ radical, which is the product of the Fenton reaction (Eq. (*B*)). A very intense ESR spectrum was observed, with the coupling constants a_N = 1.56 mT, a_H^{β} = 1.86 mT, ascribed to the DMPO–CO_2^- spin adduct[15]. The CO_2^- radicals arise from the photochemical decomposition of $[Fe(C_2O_4)_3]^{3-}$ (Eq. (*D*)).

Thermal Hydroxylation

Table I demonstrates that hydroxylation of salicylic acid occurs also in the absence of electron donors (ascorbic acid), in the mere presence of Fe(II)$EDTA^{2-}$ and oxygen. The yields of the hydroxylated products with respect to initial salicylic acid concentration are as high as 14%. As in the case of hydroxylation of salicylic acid by hydrogen peroxide in the presence of iron(II) (ref.[16]), the 2,3- to 2,5-dihydroxybenzoic acid con-

TABLE I
Thermal hydroxylation of salicylic acid to 2,5- and 2,3-dihydroxybenzoic acids. Initial concentrations (10^{-3} mol dm^{-3}): dioxygen 0.28, Fe(II) 1.3, EDTA 1.6; pH 7.1, t = 21 ± 1 °C. Concentration of salicylic acid c_{SA}, concentrations of products $c_{2,5}$, $c_{2,3}$, all in mol dm^{-3}

$c_{SA} \cdot 10^4$	$c_{2,5} \cdot 10^5$	$c_{2,3} \cdot 10^5$	Y^a, %	Remark
7.15	5.0	5.0	14.0	–
6.98	5.2	5.0	14.8	–
6.16	0.4	0.3	1.2	7.1 mM thiourea
7.19	0.9	0.9	2.6	18 mM HCOONa
6.99	0.6	0.5	1.6	36 mM HCOONa
7.56	0.6	0.6	1.6	49 mM ethanol
7.23	0	0	0	0.25 M ethanol
7.39	4.2	4.4	11.6	≈ $3 \cdot 10^{-6}$ M SOD
7.42	1.2	1.1	3.1	$3 \cdot 10^{-6}$ M catalase
6.99	5.0	5.1	14.4	$4 \cdot 10^{-6}$ M albumin
7.15	0.7	3.2	5.5	pH 3.6
7.09	4.3	4.4	12.2	pH 8.0
7.08	3.8	4.3	11.4	pH 9.0
7.12	0	0	0	$[Fe(CN)_6]^{4-}$ instead of $FeEDTA^{2-}$

[a] Reaction yield, i.e. number of moles of hydroxylated products with respect to the initial concentration of salicylic acid.

centration ratio increases from 1 : 1 in neutral solutions to 4 : 1 in acid solutions. The product concentration ratio of 1 : 1 (pH 7.1) was also found for the hydroxylation of salicylic acid with hydrogen peroxide in the absence of oxygen. Noteworthy is the effect of the enzyme catalase, which lowers the reaction yield by 79% while the ratio of the hydroxylated products remains constant.

The enzyme superoxiddismutase (SOD), which efficiently catalyzes dismutation of the superoxide anion-radical $O_2^{\bullet -}$ to oxygen and hydrogen peroxide, affects the reaction yield negligibly. The protein albumin at concentrations comparable to those of catalase does not change the reaction yield, which gives evidence that the effect of catalase consists in a rapid nonradical decomposition of hydrogen peroxide and is not due to the protein part of the enzyme, which might act as a radical scavenger. Hence, hydrogen peroxide formed by oxidation of Fe(II)EDTA^{2-} acts as a hydroxylating agent, although parallel hydroxylation by dioxygen is not impossible; catalase reduces the reaction yield but does not stop the reaction completely. When salicylic acid was only added after the quantitative oxidation of Fe(II)EDTA^{2-} by dioxygen, the resulting concentration of hydroxylated products was hundredfold lower than in experiments where the substrate was present from the very beginning. These low yields are due to oxidation of EDTA in the course of which H_2O_2 and OH$^\bullet$ radical are consumed. (Rate constant for the reaction of OH$^\bullet$ with EDTA $k_{OH} = 2.8 \cdot 10^9$ dm^3 mol^{-1} s^{-1}, ref.[17]).

The use of radical scavengers is an indirect method enabling to assess the presence of OH$^\bullet$ radicals in the reaction system. The reaction yield is reduced by 92% in the presence of 7 mmol dm^{-3} thiourea ($k_{OH} = 4.7 \cdot 10^9$ dm^3 mol^{-1} s^{-1}; ref.[18]) and by 82% in the presence of 1.8 mmol dm^{-3} sodium formate ($k_{OH} = 2.7 \cdot 10^9$ dm^3 mol^{-1} s^{-1}; ref.[18]). Hydroxylation of salicylic acid does not occur in the presence of 250 mmol dm^{-3} ethanol ($k_{OH} = 0.72 \cdot 10^9$ dm^3 mol^{-1} s^{-1}; ref.[18]). Since the rate of reaction of the OH$^\bullet$ radicals with salicylic acid is high (for benzoic acid, $k_{OH} = 4.3 \cdot 10^9$ dm^3 mol^{-1} s^{-1}; ref.[18]), a high concentration of the scavenger is necessary.

The aromatic aminoacid phenylalanine is hydroxylated to tyrosine with the yield of 2.2%, which is decreased in the presence of OH$^\bullet$ radical scavengers.

The Role of Hydrogen Peroxide

When passing from the photochemical reaction to the thermal one, the system is simplified to the 3 components: iron(II), dioxygen, and substrate, and the originally catalytic (photocatalytic) reaction becomes stoichiometric. Thus, the effects of enzymes (catalase and SOD) and OH$^\bullet$ radical scavengers can be investigated more reliably.

A reactive superoxide type intermediate $L_kFe^{2+}O_2$ occurs in the reaction of the L_kFe^{2+} complex with dioxygen[5,10]. The primary product can be[19] O_2^- (Eq. (*E*)), which in aqueous solution dismutates rapidly to H_2O_2.

$$L_kFe^{2+} + O_2 \longrightarrow L_kFe^{2+}O_2 \longleftrightarrow L_kFe^{3+}O_2^- \longrightarrow L_kFe^{3+} + O_2^- \qquad (E)$$

An alternative process, which is considerably more probable, consists in the transfer of 2 electrons from Fe(II) to dioxygen in the $L_kFe^{2+}O_2Fe^{2+}L_k$ binuclear complex[10], whereby hydrogen peroxide is formed:

$$L_kFe^{2+} + O_2 \longrightarrow L_kFe^{3+}O_2^- \xrightarrow{L_kFe^{2+}} L_kFe^{3+}O_2^-\,Fe^{3+}L_k \xrightarrow{2\,H^+} 2\,L_kFe^{3+} + H_2O_2\,. \qquad (F)$$

Similarly, L_kCu^+ complexes form the short-lived adduct $L_kCu^+O_2$ which decomposes[20] – according to conditions – either to O_2^- (one-electron mechanism) or H_2O_2 (two-electron mechanism).

The standard potential of E^0(Fe(III)/Fe(II)) = 0.77 V vs NHE (ref.[14]) shifts to more negative values in the presence of complexing agents. The decrease in the Fe(III)/Fe(II) potential to a value below 0.281 V vs NHE, which corresponds to the two-electron reduction of dioxygen to hydrogen peroxide at pH 7, favours the reduction of O_2 to H_2O_2. In the studied thermal system, Fe(II) and Fe(III) are present as EDTA complexes (log K_1 is 14.33 for Fe(II) and 24.23 for Fe(III), where E^f(FeEDTA$^-$/FeEDTA^{2-}) = 0.12 V vs NHE) or as complexes with salicylic acid (log K_2 = 11.25 for Fe(II) and log K_3 = 36.80 for Fe(III); ref.[14]). On the other hand, in the presence of $[Fe(CN)_6]^{4-}$ ($E^f([Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-})$ = 0.55 V vs NHE, ref.[14]) hydroxylation does not proceed. Hydrogen peroxide is the product of oxidation of ascorbic acid by dioxygen catalyzed by Cu(II) and Fe(III) (refs[21–23]) and has also been evidenced in the Udenfriend system[9].

The inhibiting effect of scavengers and the fact that none of the hydroxylation products is preferred (Table I) supports the concept of OH$^\bullet$ radical formation (Eq. (*B*)). The role of $L_kFe(IV)=O$ is supposed to be associated with the reaction selectivity[24]. If the catalyst L_kFe^{2+} fails to be recovered, the reaction proceeds stoichiometrically and is terminated by the oxidation of all L_kFe^{2+} to L_kFe^{3+} (Eq. (*G*)):

$$O_2 \xrightarrow{L_kFe^{2+}} H_2O_2 \xrightarrow[L_kFe^{2+}]{\text{substrate}} \text{hydroxylation products}\,. \qquad (G)$$

The catalytic cycle is closed by reduction of L_kFe^{3+} to L_kFe^{2+}. The reduction proceeds either thermally e.g. with ascorbic acid in the Udenfriend system[6] or photochemically as in the system with $[Fe(C_2O_4)_3]^{3-}$ or other iron(III) complexes[25,26].

REFERENCES

1. McMurry T. J., Groves J. T. in: *Cytochrome P-450: Structure, Mechanism and Biochemistry* (P. R. Ortiz de Montellano, Ed.), p. 1. Plenum Press, New York 1986.
2. Nishiki M., Satoh T., Sakurai H.: J. Mol. Catal. *62*, 79 (1990).
3. Carrier M.-N., Scheer C., Gouvine P., Bartoli J.-F., Battioni P., Mansuy D.: Tetrahedron Lett. *31*, 6645 (1990).
4. Funabiki T., Toyoda T., Ishida H., Tsujimoto M., Ozawa S., Yoshida S.: J. Mol. Catal. *61*, 235 (1990).
5. Ohkubo K., Ishida H., Sagawa T., Miyata K., Yoshinaga K.: J. Mol. Catal. *62*, 107 (1990).
6. Udenfriend S., Clark C. T., Axelrod J., Brodie B. B.: J. Biol. Chem. *208*, 731 (1954).
7. Ito S., Ueno K., Mitarai A., Sasaki K.: J. Chem. Soc., Perkin Trans. 2 *1993*, 255.
8. Halliwell B., Gutteridge J. M. C.: FEBS Lett. *128*, 347 (1981).
9. Schwertnerova E., Wagnerova D. M., Veprek-Siska J.: Collect. Czech. Chem. Commun. *44*, 2893 (1979).
10. Bielski B. H. J. in: *Oxygen Radicals in Biology and Medicine* (M. G. Simic, K. A. Taylor, J. F. Ward and C. von Sonntag, Eds), p. 123. Plenum Publishing Corp., New York 1989.
11. Sugimoto H., Sawyer D. T.: J. Am. Chem. Soc. *106*, 4283 (1984).
12. Murov S. L.: *Handbook of Photochemistry*, p. 119. Dekker, New York 1973.
13. Hennig H., Rehorek D., Archer R. D.: Coord. Chem. Rev. *61*, 1 (1985).
14. Dean J. A., Ed.: *Lange's Handbook of Chemistry*, 13th ed. McGraw–Hill Book Company, New York 1985.
15. Zhang Y.-K., Lu D.-H., Xu G.-Z.: Z. Naturforsch. *45*, 1075 (1990).
16. Grinstead R. R.: J. Am. Chem. Soc. *82*, 3472 (1960).
17. Bhattachargga S. N., Kundu K. P.: Int. J. Radiat. Phys. Chem. *4*, 31 (1972).
18. Halliwell B., Gutteridge J. M. C.: *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford 1985.
19. Michelson A. M. in: *Superoxide and Superoxide Dismutases* (A. M. Michelson, J. M. McCord and I. Fridovich, Eds), p. 77. Academic Press, London 1977.
20. Goldstein S., Szapski G., van Eldik R., Cohen H., Meyerstein D.: J. Phys. Chem. *95*, 1282 (1991).
21. Khan M. M.: Oxid. Commun. *9*, 105 (1986).
22. Schwertnerova E., Wagnerova D. M., Veprek-Siska J.: Collect. Czech. Chem. Commun. *41*, 2463 (1976).
23. Wagnerova D. M., Schwertnerova E., Veprek-Siska J.: Collect. Czech. Chem. Commun. *41*, 2473 (1976).
24. Groves J. T., Puy M. V. D.: J. Am. Chem. Soc. *96*, 5274 (1974).
25. Klementova S., Wagnerova D. M.: Marine Chem. *30*, 89 (1990).
26. Klementova S., Wagnerova D. M.: Collect. Czech. Chem. Commun. *59*, 1066 (1994).

Translated by P. Adamek.