Oxidation of Sulfides by Peroxidases. Involvement of Radical Cations and the Rate of the Oxygen Rebound Step

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Horseradish peroxidase (HRP) is a hemoprotein peroxidase capable of catalyzing the oxidation of a large variety of organic compounds. The reaction mechanism generally involves the sequential electron abstraction from two substrate molecules, whereby the ferryl porphyrin radical cation ($Por^{\bullet+}-Fe^{IV}=O$), compound **I**, formed by reaction of H_2O_2 with the ferric enzyme, is reduced first to $Por-Fe^{IV}=O$, known as compound **II**, and then to the resting ferric state.[1−3] Typical of these oxidations is that HRP does not transfer the ferryl oxygen to the substrates. Thus the finding that HRP can catalyze the oxidation of sulfides to sulfoxides by a 2-e process involving the ferryl oxygen transfer to the S atom has recently raised great interest.[4−11]

Two mechanisms have been proposed to explain this observation, both of which involve, as the initial step, electron transfer from the sulfide to compound **I** to give the radical cation $R_2S^{\bullet+}$ (eq 1).[8,10] The mechanisms differ with respect to the conversion

$$Por^{\bullet+}-Fe^{IV}=O\ (\mathbf{I}) + R_2S \rightarrow Por-Fe^{IV}=O\ (\mathbf{II}) + R_2S^{\bullet+} \quad (1)$$

of $R_2S^{\bullet+}$ to sulfoxide. In one, it is suggested that sulfides bind to the enzyme at a site different from where the classical HRP substrates bind, enabling the transfer of oxygen from compound **II** to the sulfide radical cation, the oxygen rebound step (eq 2).[8] In the other mechanism,[10,11] it is proposed that a hydroxyl

$$Por-Fe^{IV}=O\ (\mathbf{II}) + {}^{\bullet+}SR_2 \rightarrow Por-Fe^{III} + O=SR_2 \quad (2)$$

radical (presumably formed after H^+ transfer to oxygen by a distal group) is released from compound **II**, which then reacts with the sulfur radical cation forming the sulfoxide.

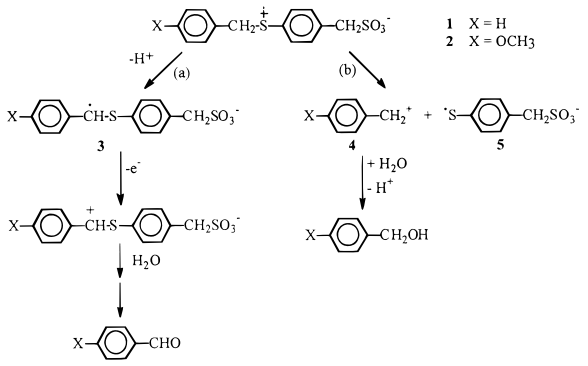
To get further insight into the mechanism of oxidation of R_2S by HRP, we have now investigated the HRP-induced oxidation of the water soluble aromatic sulfides **1** and **2**[12] which can form radical cations capable of undergoing C−H and C−S bond cleavage,[13] reactions that lead to products different from sulfoxides. The results of this study are presented here together with a pulse radiolysis investigation of the radical cations $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$. For comparison were investigated the oxidations of **1** and **2** with chloroperoxidase (CPO).

Table 1. HRP Catalyzed Oxidations of Sulfides **1** and **2** by H_2O_2

Substrate	Products and yields[a]		
	X-⟨⟩-CH_2-SO-⟨⟩-CH_2SO_3K	X-⟨⟩-CHO	X-⟨⟩-CH_2OH
1 (X = H)	1.06 (0.48)[b]	0.10 (<0.02)	0.14 (<0.02)
2 (X = OCH_3)	1.14 (0.54)	0.10 (<0.02)	0.20 (<0.02)

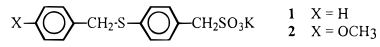
[a] Absolute yields in μmol. Average of at least two determinations. [b] Values in parentheses are referred to the reaction with H_2O_2 in the absence of the enzyme. No products are formed when H_2O_2 is omitted.

Scheme 1



1 and **2** were reacted with HRP using standard procedures.[9,11] For example, the sulfide (20 μmol) and HRP (Sigma, type VI) (0.05 μmol) in 3 mL of 0.1 M phosphate buffer, pH 6, at 25 °C were magnetically stirred. H_2O_2 (20 μmol) was added in 10 aliquots at 10 min intervals. The reaction was quenched with sodium sulfite 2 h after the first addition of H_2O_2 and the solution extracted with CH_2Cl_2. The products in the organic phase (alcohol and aldehyde) were quantitatively determined by GC, whereas the sulfoxide in the aqueous phase was analyzed by HPLC on a reversed-phase column (C8) using 0.1% (v/v) trifluoroacetic acid in MeOH−H_2O (1:1 v/v) as the mobile phase. As internal standards were used 4-MeO- or 4-HOC_6H_4-$COCH_3$ (GC or HPLC).

The reaction products (Table 1) are the sulfoxides of **1** and **2** (major products) and benzaldehyde and benzyl alcohol from **1** and 4-methoxybenzaldehyde and 4-methoxybenzyl alcohol from **2**. The aldehydes and the alcohols are typical products of fragmentation of the radical cations, $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$, from which they derive by C−H deprotonation to form the benzyl radicals **3** and by C−S bond cleavage to produce the benzyl carbocations **4**. Benzaldehyde is obtained by oxidation of **3** followed by hydrolysis (Scheme 1, X = H, OCH_3, path a), a path resembling that suggested for the N-dealkylation of amines by HRP.[14] The benzyl alcohols derive from the reaction with water of the benzyl carbocations (path b).[15] The sulfoxides are conceivable as products of an oxygen rebound step, eq 2.



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(12) Compounds **1** and **2** were obtained by the reaction of benzyl and 4-methoxybenzyl chlorides with 4-mercaptobenzyl sulfonic acid[12a,b] in the presence of anhydrous potassium carbonate, in refluxing acetone.[12c] (a) Beringer, F. M.; Falk, R. A. *J. Am. Chem. Soc.* **1959**, *81*, 2977. (b) Kawai, H.; Sakamoto, F.; Taguchi, M.; Kitamura, M.; Sotomura, M.; Tsukamoto, G. *Chem. Pharm. Bull.* **1991**, *39*, 1422. (c) Baciocchi, E.; Intini, D.; Piermattei, A.; Rol, C.; Ruzziconi, R. *Gazz. Chim. Ital.* **1989**, *119*, 649.
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In order to check these ideas, $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$ were produced by *chemical* oxidation using $SO_4^{\bullet-}$ (eq 4). Thus, a deoxygenated solution of **1** or **2** (0.1 mM), $K_2S_2O_8$ (2 mM), and *t*-BuOH (0.1 M) at pH 4 was irradiated with a ^{60}Co γ source (product analysis) or with 0.4 μs pulses of 3 MeV electrons delivered by a van de Graaff accelerator (pulse radiolysis). $SO_4^{\bullet-}$ was obtained via the radiation-chemically generated hydrated electron, e_{aq}^- (eq 3). The products from **1** were benzyl alcohol and benzaldehyde in a 0.9:1 ratio; those from **2** were the corresponding 4-methoxy compounds in a 2.5:1 ratio (the sulfoxides of **1** and **2** were not produced).[16] These ratios are similar to those from the enzymatic oxidation (see Table 1).

$$e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{\bullet-} + SO_4^{2-} \quad (3)$$

$$SO_4^{\bullet-} + \mathbf{1}\ (\mathbf{2}) \rightarrow SO_4^{2-} + \mathbf{1}^{\bullet+}\ (\mathbf{2}^{\bullet+}) \quad (4)$$

With pulse radiolysis, on reaction of **1** and **2**, strong absorptions at 320 and 520−600 nm were observed 8 μs after the initiating pulse (as seen in Figure 1 for the case of **2**). The transients are assigned to the corresponding radical cations, in the monomeric form.[17] Both $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$ decay by a first-order process with a rate constant of 2.6×10^3 s^{-1} ($\mathbf{1}^{\bullet+}$) and 3.6×10^3 s^{-1} ($\mathbf{2}^{\bullet+}$), respectively.[18] In the inset b of Figure 1, it is shown that the decay of the radical cation is accompanied by an increase of conductance, which is due to the production of H^+. The radical cation undergoes two decomposition reactions: deprotonation with formation of the benzyl-type radical **3** (X = H, OCH_3), which has an absorption band at 360 nm[19] (see Figure 1, 220 and 750 μs after the pulse) and which can be scavenged by O_2 (see inset d), and cleavage of the C−S bond with formation of the arylthiyl radical **5**, which is recognizable by the absorptions at 310 and 420−540 nm observed after 220 and 750 μs.[20] The lifetimes of the radical cations or of **5** were not influenced by O_2.

If in the enzymatic reaction the sulfide radical cations undergo partitioning between oxygen rebound and fragmentation, as illustrated in Scheme 2, it is possible to estimate the rate of the oxygen rebound step, k_r, from the overall fragmentation rate (k_f) of the radical cation and the product distribution. The fragmentation rate in the *absence* of enzyme is that measured by the pulse radiolysis technique, and it is assumed that it is the same in the *presence* of the enzyme.

Then, from the k_f-value and the ratio of the yield of sulfoxide and that of the fragmentation products in the enzymatic reaction (see Table 1), the rate constant of the oxygen rebound process leading to sulfoxide from the radical cation (see Scheme 2) follows as $k_r = k_f[ArCH_2(Ar')S{=}O]/[ArCH_2OH + ArCHO] = 6.3 \times 10^3$ and 7.2×10^3 s^{-1} for $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$, respectively. Of course, these numbers have to be considered with some caution,

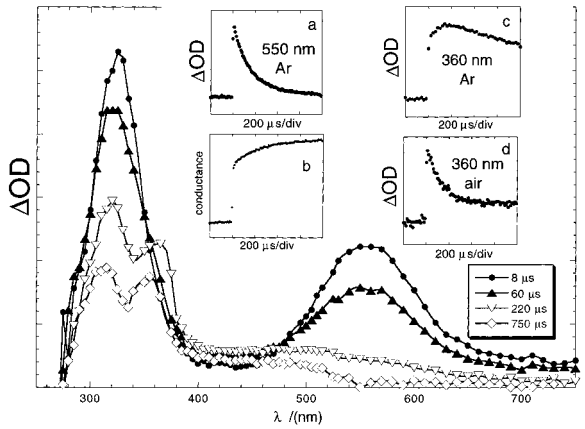
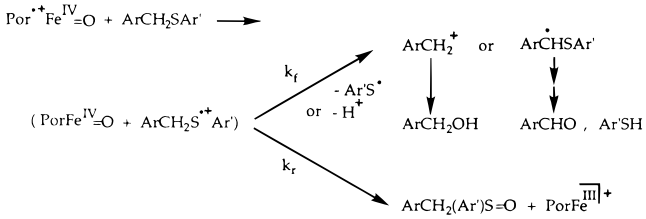


Figure 1. Absorption spectra recorded on pulse radiolysis (dose = 4 Gy) of an Ar-purged aqueous solution (pH 4.0) of **2** (0.1 mM), $K_2S_2O_8$ (2 mM), and *t*-BuOH (0.1 M) at the times after the pulse as indicated by the symbols. Inset a shows the decay of $\mathbf{2}^{\bullet+}$ at 550 nm; inset b shows the rise of conductance resulting from the decay of $\mathbf{2}^{\bullet+}$ at pH 4.5 (dose = 1.6 Gy); inset c shows the formation and decay (by radical−radical reaction) of the carbon-centered radical **3** (X = OMe) at 360 nm, in the absence of O_2. From inset d it is evident that **3** is scavenged by O_2. The decay at 360 nm has the same rate as that of $\mathbf{2}^{\bullet+}$ at 550 nm which shows that $\mathbf{2}^{\bullet+}$ has a weak absorption at 360 nm.

Scheme 2



in view of the differences in microenvironment between the chemical and the enzymatic systems.

In order to compare HRP with another enzyme, we studied the oxidation of **1** and **2** as catalyzed by CPO.[21] The CPO-induced oxidations lead exclusively to the formation of sulfoxides; i.e., there was no evidence for the formation of the fragmentation products of the radical cation. Clearly, if sulfide radical cations are at all involved in this reaction, the oxygen rebound step must be much faster with CPO than with HRP, a conclusion in line with the greater accessibility of the ferryl oxygen in the former enzyme which makes possible a direct oxygen transfer to the radical cation.[22] However, the possibility cannot be excluded that with CPO the O is transferred to the sulfide without the intervention of free radical intermediates, a path that is likely in view of the recent report suggesting an oxygen insertion mechanism in CPO-induced alkane hydroxylation.[23]

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(15) The reaction of the (incipient) benzyl cation with water may be concerted with the fragmentation of the C−S bond.

(16) The benzyl alcohols and benzaldehydes were also formed on chemical oxidation with $Co^{III}W_{12}O_{40}^{5-}$.

(17) The same signal was observed when the aromatic sulfide radical cation was generated by reaction of e_{aq}^- with the sulfoxide (cf. Engman, L.; Lind, J.; Merényi, G. *J. Phys. Chem.* **1994**, *98*, 3174), i.e., in the absence of any sulfide which could possibly lead to the formation of a *dimeric* radical cation.

(18) Due to second-order contributions, the measured rate constants are somewhat dose dependent. To correct for this effect, dose variations were performed, on the basis of which the rate constants for the decomposition of $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$ were extrapolated to dose = 0.

(19) The benzyl radicals **3** can be selectively produced by reaction of **1** or **2** with $O^{\bullet-}$ at pH 13.5 or by reaction of $\mathbf{1}^{\bullet+}$ and $\mathbf{2}^{\bullet+}$ with a high concentration of base such as OH^- or HPO_4^{2-} (Ioele, M.; Steenken, S.; Baciocchi, E. Unpublished results).

(20) The spectrum of the authentic arylthiyl radical **5** was obtained by oxidizing 4-$HSC_6H_4CH_2SO_3^-$ with Tl^{2+} at pH 3.6, using pulse radiolysis (N_2O-saturated aqueous solution, 1 mM Tl_2SO_4, 0.1 mM $HSC_6H_4CH_2SO_3K$).

(21) The sulfide (20 μmol) and CPO (Sigma) (0.3 nmol) were magnetically stirred in 5 mL of 0.1 M citrate buffer, pH 5, at 25 °C. H_2O_2 (20 μmol) was added in 5 aliquots at 5 min interval. The reaction was quenched with sodium sulfite 45 min after the first addition of H_2O_2 and the solution extracted with CH_2Cl_2. No products were detected by GC analyses of the organic phase, whereas the sulfoxides (2 and 5.4 μmol from **1** and **2**, respectively) were observed and quantitatively determined by HPLC analyses of the aqueous phase.

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