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The activation of molecular oxygen by horseradish peroxidase with sodium sulfite†

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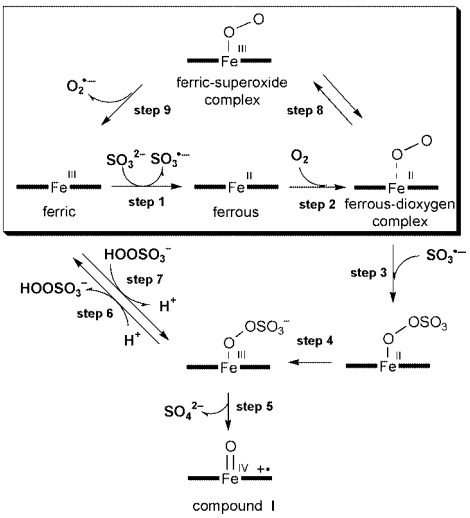
Horseradish peroxidase (HRP) utilizes molecular oxygen (O_2) with sodium sulfite (Na_2SO_3) to oxidize thioanisole and styrene at the exterior of the heme pocket.

Horseradish peroxidase (HRP) bearing iron protoporphyrin (IX) as the prosthetic group normally utilizes hydrogen peroxide (H_2O_2) as an oxidant to generate an oxoferryl species ($O{=}Fe^{IV}$) paired with porphyrin radical cation, so-called compound **I**.[1] Compound **I** is reduced back to the ferric state by either sequential one-electron transfer from typical phenolic substrates such as guaiacol or by ferryl oxygen transfer to thioethers.[2–4] Since HRP is known to react with racemic hydroperoxides to afford chiral hydroperoxides and alcohols in high enantioselectivity, the enzyme is also used for kinetic resolutions.[5–9] In contrast to P450 monooxygenases, HRP does not efficiently activate O_2 with a reductase and NADH or NADPH because the peroxidase does not mediate the electron transfer from external electron sources on the protein surface to the heme iron buried inside.[10] However, the direct electron transfer from sulfite (SO_3^{2-}) to the ferric heme iron would produce $SO_3^{\cdot}$ (step 1 in Scheme 1), which could initiate the activation of O_2 to oxidize the substrates.[11,12] In order to explore transition metal promoted oxidative reactions with sulfite, we have investigated one- and two-electron oxidation by HRP with SO_3^{2-} and O_2.

Thioanisole is oxidized to methyl phenyl sulfoxide by HRP in the presence of sodium sulfite and ambient O_2 at a rate more than four times faster than the value obtained in the incubation with H_2O_2 as an oxidant (Table 1). Not more than a trace amount of the sulfoxide product is detected when the reaction is performed under anaerobic conditions. The results clearly indicate that HRP together with sulfite can activate molecular oxygen to produce a potential oxidant for the monooxygenation reaction. Interestingly, sulfoxidation with SO_3^{2-} and O_2 does not proceed enantioselectively as observed in the reaction with H_2O_2 (Table 1); therefore, compound **I** does not seem to be a catalytic species in the HRP–SO_3^{2-}–O_2 system (*i.e.* pathway step 3 → 4 → 5 in Scheme 1 is not dominant). Since the oxidation of thioanisole with monoperoxysulfate (HSO_5^-) has been found to afford a racemic mixture of sulfoxide in the presence or absence of HRP, we speculated that monoperoxysulfate released from the heme pocket would be involved in sulfoxidation with sulfite and molecular oxygen.

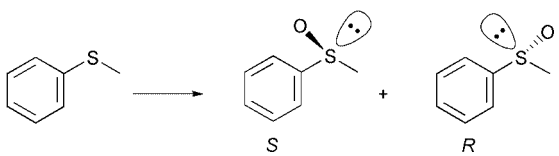
In order to investigate the mechanism further, we have performed the sulfoxidation reaction in the presence of superoxide dismutase (SOD) and catalase. The reaction is subject to significant inhibition (97%) although SOD and catalase do not decelerate the chemical sulfoxidation by monoperoxysulfate (Table 2). By contrast, the rate of sulfoxidation is reduced by < 18% in the presence of $OH^{\cdot}$ radical scavengers such as methanol or *tert*-butyl alcohol and $SO_4^{\cdot-}$ radical quenchers like ethanol (Table 2). The results imply that (a) superoxide ($O_2^{\cdot-}$) is involved in the production of monoperoxysulfate and (b) the lack of inhibition observed in the presence of ethanol, methanol or *tert*-butyl alcohol argues against the involvement of $SO_4^{\cdot-}$ or $OH^{\cdot}$ radicals in the catalysis. The ferrous–dioxygen and ferric–superoxide complex of HRP are in equilibrium (step 8 in Scheme 1). Thus, the recombination of $O_2^{\cdot-}$ and $SO_3^{\cdot-}$ generated in step 9 and 1 (Scheme 1), respectively, could produce SO_5^{2-}, which is subsequently protonated to produce monoperoxysulfate (HSO_5^-). The release of HSO_5^- from the intermediate by Fe–O



Scheme 1 Reaction scheme for the HRP–SO_3^{2-}–O_2 system. The catalytic cycle postulated by our results is indicated inside the square; $SO_3^{\cdot-}$ and $O_2^{\cdot-}$ generated by step 1 and 9, respectively, would mediate the oxidation reaction outside of the active site under the conditions described here.

† Electronic supplementary information (ESI) available: plots of pH *vs.* rate of sulfoxidation. See http://www.rsc.org/suppdata/cc/b1/b104529f/

Table 1 Oxidation of thioanisole[a]



Protein	Oxidant	Initial rate/ turnover min^{-1}	%ee
HRP	H_2O_2	1.1	77[b]
HRP	sulfite and O_2	1.2	0

[a] The reaction mixture containing HRP (5 μM) and thioanisole (2 mM) was incubated with either H_2O_2 (0.6 mM) or sodium sulfite (0.6 mM) at 25 °C in sodium phosphate buffer (50 mM, pH 7.0). The sulfoxide product extracted with CH_2Cl_2 was analyzed by HPLC equipped with a Dicel OD chiral column as reported in ref. 4. The reported values are the average of two independent experiments. For anaerobic experiments, the vessel containing the reaction mixture was frozen, evacuated and then filled with nitrogen. The procedure was repeated three times to remove dissolved oxygen. Sulfoxidation was then performed under a nitrogen atmosphere. [b] The *S* isomer is the major product.

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Table 2 Inhibition of thioanisole oxidation by additives[a]

Additive	Relative activity (%)
—[b]	100
SOD and catalase[c]	3
Methanol[d]	96
Ethanol[d]	82
tert-Butyl alcohol[d]	96

[a] Reactions were conducted with HRP (5 μM), thioanisole (2 mM), sodium sulfite (0.6 mM) and additive(s) in sodium phosphate buffer (50 mM, pH 7.0) at 25 °C for 10 min. The reported values are the average of two independent experiments. SOD and catalase did not inhibit chemical oxidation of thioanisole by monoperoxysulfate (0.6 mM). [b] No additive. [c] SOD (10 units) and catalase (11 units) added to the reaction mixture. [d] The concentration of additive is 25 mM.

bond cleavage (step 6 in Scheme 1) is excluded because superoxide is not involved in the catalytic cycle (step 1 → 2 → 3 → 6 in Scheme 1) and the observed inhibition in the presence of SOD and catalase can not be rationalized.

Plots of sulfoxidation rate *vs.* pH examined in the range pH 5–10 reveal that the reaction with sulfite does not proceed below pH 5, and the optimum pH is found to be 7 (see ESI†). The trend here is similar to that observed for nickel-catalyzed oxidative deamination with sulfite under aerobic conditions[13] but differs from the pH profile for the HRP–H_2O_2 system, which can produce sulfoxide even below pH 6. The result is consistent with our hypothesis that the oxidation mechanism is altered when SO_3^{2-}–O_2 instead of H_2O_2 is utilized.

Styrene and guaiacol oxidations by HRP with sulfite and oxygen provide further support for the proposed reaction scheme (Scheme 1). In contrast to sulfoxidation, the epoxidation reaction by compound **I** requires interactions of the two vinyl carbons with a ferryl oxygen atom. Since the active site of HRP is sterically hindered, it was previously reported that compound **I** of HRP could not efficiently oxidize styrene.[2,4] However, styrene oxide is detected in the mixture of HRP–styrene–SO_3^{2-}–O_2 (Table 3) although the rate of oxidation is slow. In addition, phenylacetaldehyde, which is normally observed as a side product of compound **I** mediated epoxidation by other hemoproteins, is not produced.[14–18] The results indicate that monooxygenation reactions in the HRP–SO_3^{2-}–O_2 system do not proceed *via* compound **I** but occur outside of the heme pocket by monoperoxysulfate. Significantly, slow one-electron oxidation of guaiacol exhibited by HRP with SO_3^{2-} and O_2 also indicates that the intermediate formation

Table 3 Epoxidation of styrene

Experiment	Protein	Oxidant	Rate
1[a]	HRP	H_2O_2	ND[c]
2[a]	HRP	Sulfite and O_2	200[b]
3[d]	—	Monoperoxysulfate	250[b]
4[e]	—	Sulfite and O_2	ND[c]

[a] Styrene (1 μL) was added to HRP (5 μM) in 0.5 mL of sodium phosphate buffer (50 mM, pH 7.0). The concentration of styrene was expected to be 17 mM, but the reaction mixture appeared to be slightly turbid. To the styrene-saturated solution, either H_2O_2 (0.6 mM) or sodium sulfite (0.6 mM) was added to initiate the reaction at 25 °C. Products were extracted with CH_2Cl_2 and analyzed by GC on a Shimadzu CBP1 capillary column. The reaction time varied from 20 to 120 min to obtain the time *vs.* epoxide formation plot. A linear relationship was observed for 120 min, and the rate was determined as the slope of the plot. The quoted values are the average of two independent experiments. [b] pmol of epoxide min^{-1}. [c] Not detected.[d] The reaction was performed with monoperoxysulfate (0.6 mM) in the absence of HRP. Since the exact concentration of monoperoxysulfate in the HRP–sulfite–O_2 solution can not be determined, direct comparison of the rates in experiments 2 and 3 would not be appropriate, however, the results indicated that the epoxide could be produced from styrene with monoperoxysulfate. [e] Oxidation was also performed with sodium sulfite (0.6 mM) with styrene aerobically in the absence of HRP, but the epoxide product was not detected.

(step 3 in Scheme 1) or/and the heterolytic cleavage of the O–O bond in Fe(III)–SO_5^{2-} species (step 5 in Scheme 1) is not an efficient process since guaiacol can not be oxidized by monoperoxysulfate but only by compound **I**. The addition of an excess of monoperoxysulfate to ferric HRP (step 7 in Scheme 1) somewhat facilitates compound **I** formation to improve the one-electron oxidation activity (4.1 turnover min^{-1}); however, the rate is 6500-fold slower than the value obtained in guaiacol oxidation with H_2O_2. It was previously reported that high valent metal–oxo species generated from water-soluble porphyrins and oxygen atom donors ($KHSO_5$, H_2O_2, … *etc.*) mediate the oxidation of alcohols, olefins or DNA,[19–23] but the active site of HRP may not be large enough to accommodate HSO_5^- as a good oxidant.

In summary, we report that HRP can utilize SO_3^{2-} and O_2 to oxidize thioanisole and styrene, but the catalytic species is not compound **I** as established in the oxidation with H_2O_2. Our mechanistic studies imply that monoperoxysulfate (HSO_5^-) generated from $O_2^{\cdot-}$ and $SO_3^{\cdot-}$ mediates the oxidation reaction outside of the heme pocket. A similar mechanism might be involved in the metalloprotein associated biological toxicity of sulfite inhaled from industrial emissions or ingested as a preservative in foods.[24]

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