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Direct Detection of the Sulfur Trioxide Radical Anion during the Horseradish Peroxidase-Hydrogen Peroxide Oxidation of Sulfite (Aqueous Sulfur Dioxide)

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SUMMARY

The ESR spectrum of $\dot{S}O_3^-$ is observed directly during the oxidation of (bi)sulfite to sulfate by horseradish peroxidase. This radical exhibits a single line at $g = 2.0031$. The $\dot{S}O_3^-$ radical can be trapped with nitrosobenzene, yielding an ESR spectrum with coupling constants $A^N = 12.3$ G, $A_p^H = A_o^H = 2.4$ G, and $A_m^H = 0.9$ G, and a g-value of 2.0053. $\dot{S}O_3^-$ is an intermediate in the two-step reduction of peroxidase Compound I by (bi)sulfite at physiological pH. At low pH, no $\dot{S}O_3^-$ is observed, which indicates a direct, one-step, two-electron reduction of Compound I. The pH at which the mechanism changes depends on the isoenzymes present. The radical reacts rapidly with oxygen as evidenced by the absence of an ESR spectrum when oxygen is present and by oxygen uptake measurements.

INTRODUCTION

Sulfur dioxide is recognized as a major air pollutant, particularly near large cities (1), while the ionized forms, bisulfite and sulfite, are found as preservatives in food and beverages. Because of the pervasiveness of these compounds in the environment, there is concern over the way in which these sulfur oxides are metabolized by both plant and mammalian systems. The over-all reaction is the oxidation of (bi)sulfite to sulfate and its eventual excretion in urine (2). Most attention has been focused on the enzyme sulfite oxidase, which oxidizes (bi)sulfite to sulfate (3). This paper focuses on another way in which this transformation may occur, namely the oxidation of (bi)sulfite by peroxidase enzymes.

In lung, sulfur dioxide is hydrated rapidly.

$$H_2O + SO_2 \rightleftarrows HSO_3^- + H^+$$

The equilibrium constant for this reaction is 1.7×10^2 moles/liter (2), and hence, at physiological pH, bisulfite predominates. The bisulfite ion is a weak acid, dissociating according to the reaction

$$HSO_3^- + H_2O \rightleftarrows H_3O^+ + SO_3^{2-}$$

with an equilibrium constant of 1.02×10^{-7} moles/liter. At pH greater than 7, the equilibrium lies to the right and sulfite predominates, although there is always an equilibrium between sulfite and bisulfite. In this paper, the term (bi)sulfite is used when it cannot be determined, or does not matter, which species is involved in a reaction.

The autoxidation of (bi)sulfite to sulfate has been studied extensively and shown to involve a free radical mechanism (4, 5). The (bi)sulfite free radical ($\dot{S}O_3^-$) produced as a result of the autoxidation is proposed to be involved in a number of reactions of biological significance: the oxidation of diphosphopyridine nucleotide (6) and methionine (7), the destruction of tryptophan (8) and β-carotene (9), the addition of $\dot{S}O_3^-$ across the double bonds of alkenes (10, 11) and of various nucleotides and nucleic acids (12), and the peroxidation of fatty acids (13) and rat liver homogenate (14), as well as the cleavage of DNA (15). This paper deals not with autoxidation of (bi)sulfite, but with the oxidation of (bi)sulfite by peroxidase enzymes; this process yields the same radical thought to be produced by autoxidation.

The initiation of (bi)sulfite oxidation by horseradish peroxidase and hydrogen peroxide in the presence of Mn^{2+} and/or phenolic compounds has been reported (4, 6-8). All of these studies suggest the formation of the (bi)sulfite radical, $\dot{S}O_3^-$, although the presence of this radical has not been proven in these systems. The mechanism by which sulfite oxidation occurs in systems containing horseradish peroxidase has been proposed to be a direct one-step, two-electron reduction of horseradish peroxidase-Compound I at acidic pH (16, 17) and two successive one-electron reductions at higher pH (17). The pH at which the one-electron mechanism becomes dominant depends on the isoenzymes present in the peroxidase preparation. If the mechanism does involve two consecutive one-electron reductions of Compound I, then the (bi)sulfite radical should be produced as an intermediate. This work reports the observation of the $\dot{S}O_3^-$ free radical in the horseradish peroxidase system with ESR and is the first observation of the $\dot{S}O_3^-$ radical anion

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in an enzymatic system. In the absence of oxygen, this free radical decays by second-order kinetics in a nearly pH-independent manner with $2k = 1.1 \times 10^9$ M^{-1} sec^{-1} (18) and is probably the most reactive free radical metabolite to be detected directly with ESR in any biological system.

MATERIALS AND METHODS

Sodium sulfite and hydrogen peroxide were American Chemical Society-certified from Fisher Scientific Company (Pittsburgh, Pa.). Catalase (thymol-free), DETAPAC,[2] and horseradish peroxidase [Type VI, Type VIII acidic isoenzyme (peroxidase C), and Type IX basic isoenzyme (peroxidase A)] were purchased from Sigma Chemical Company (St. Louis, Mo.). Superoxide dismutase was obtained from Diagnostic Data Inc. (Mountain View, Calif.). Nitrosobenzene was purchased from Aldrich Chemical Company (Milwaukee, Wisc.).

All ESR spectra were recorded with a Varian E-104A instrument at 9.1 GHz, using a TM_{110} cavity with aqueous flat cell at room temperature. The g-value measurements were made relative to a solution of Fremy's salt (g = 2.0055) in a capillary tube attached to the side of the flat cell. Oxygen uptake measurements were made at room temperature with a Yellow Springs Instrument Company oxygen monitor (Model 53).

Typical incubations for both ESR and oxygen uptake work were 0.03 milliformula weights of Na_2SO_3, 0.03 micromoles of H_2O_2, and 0.75 mg of horseradish peroxidase in a total volume of 3 ml. The DETAPAC concentration was 1 mM when it was used. Catalase was used at a maximal concentration of 30,000 units/ml and superoxide dismutase at a maximal concentration of 40 μg/ml.

RESULTS

A short-lived radical with a single-line ESR spectrum was observed at pH 8.6 using Type VI horseradish peroxidase (Fig. 1), at pH 8.6 using the Type IX basic isoenzyme, and at pH 7.4 and pH 8.6 using the Type VIII acidic isoenzyme. The signal was not observed at pH 7.4 with the Type VI horseradish peroxidase, which contains no acidic isoenzymes. The species giving rise to this line was identified as the $\dot{S}O_3^-$ radical based on comparison of the experimental g-value (2.0031) with the reported g-values of the sulfur trioxide anion free radical [2.00306 (19), 2.0030 (11), and 2.0033 (10)]. After a steady-state concentration was obtained, which lasted for a few minutes, the $\dot{S}O_3^-$ signal disappeared suddenly, as would be expected for the decay of an unstable radical which is no longer being formed.

The radical was also spin-trapped with nitrosobenzene, yielding an ESR spectrum with coupling constants A^N = 12.3 G, $A_p^H = A_o^H$ = 2.4 G, and A_m^H = 0.9 G, and a g-value of 2.0053 as compared with reported hyperfine splitting constants for the nitrosobenzene-$\dot{S}O_3^-$ adduct of A^N = 12.21 G, $A_o^H = A_p^H$ = 2.38 G, and A_m^H = 0.90 G (20). The addition of $\dot{S}O_3^-$ across the carbon-carbon double bond of fatty acids or the carbon-sulfur double bond of 4-thiouridine (12) differs from the addition across the nitrogen-oxygen double bond of nitrosobenzene only in that the resulting radicals are not stable. This spin-trapping experiment does not provide conclusive evidence for the production of $\dot{S}O_3^-$, since the nitrosobenzene-$\dot{S}O_2^-$ adduct yields essentially the same spectrum (20). However, the direct observation does prove conclusively the presence of $\dot{S}O_3^-$, since the g-value of $\dot{S}O_3^-$ is far removed from that of $\dot{S}O_2^-$ [2.0058 (11)] or other sulfur/oxygen free radicals such as $\dot{S}O_4^-$ [2.0125 (21)]. The pH of solutions used in this work exceeds the pK_a of the $H\dot{S}O_3$ radical [pK_a = 4.52, (22)], and consequently the ESR spectrum does not have a proton hyperfine coupling.

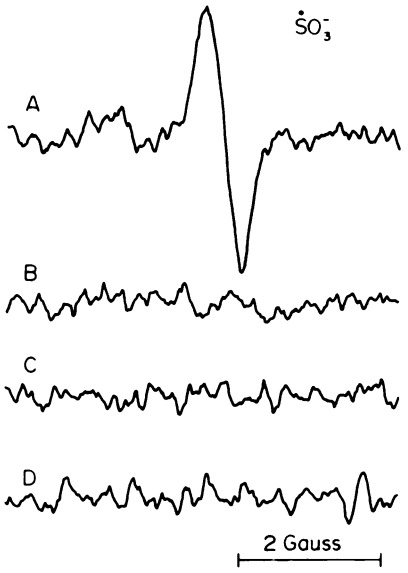

FIG. 1. *ESR spectra of the HRP/H_2O_2/sulfite system*
A, 10 mM Na_2SO_3, 10 μM H_2O_2, and Type VI HRP (0.25 mg/ml, 83 units/ml) in pH 8.6 boric acid/sodium borate buffer; B, 10 mM Na_2SO_3 and 10 μM H_2O_2 in buffer; C, 10 mM Na_2SO_3 and HRP (0.25 mg/ml) in buffer; D, 10 μM H_2O_2 and HRP (0.25 mg/ml) in buffer. For all spectra the modulation amplitude was 0.66 G, time constant 1 sec, sweep time 4 min, power 20 mW, and gain 1.6×10^6.

As can be seen from Fig. 1, all of the components of the system—(bi)sulfite, H_2O_2, and horseradish peroxidase—had to be present in order to observe the $\dot{S}O_3^-$ spectrum. The following equations account for radical formation in this system.

$$\text{HRP} \xrightarrow{H_2O_2} \text{HRP-Compound I} \quad (1)$$

$$\text{HRP-Compound I} + \text{(bi)sulfite} \rightarrow \text{HRP-Compound II} + \dot{S}O_3^- \quad (2)$$

$$\text{HRP-Compound II} + \text{(bi)sulfite} \rightarrow \text{HRP} + \dot{S}O_3^-. \quad (3)$$

The $\dot{S}O_3^-$ signal was totally inhibited by the use of heat-denatured horseradish peroxidase, implying that enzymatic activity, not merely the presence of heme, is necessary for detectable radical formation.

At higher concentrations of H_2O_2, there is a reaction with (bi)sulfite which produces $\dot{S}O_3^-$ non-enzymatically (10); this was also observed in our work.

$$H_2O_2 + \text{(bi)sulfite} \rightarrow \dot{S}O_3^- + \dot{O}H + OH^- \text{ (or } H_2O) \quad (4)$$

In all of the work presented here, the H_2O_2 concentration was sufficiently low that radical production via this chemical reaction was nearly undetectable (Fig. 1B).

[2] The abbreviations used are: DETAPAC, diethylenetriaminepentaacetic acid; HRP, horseradish peroxidase.

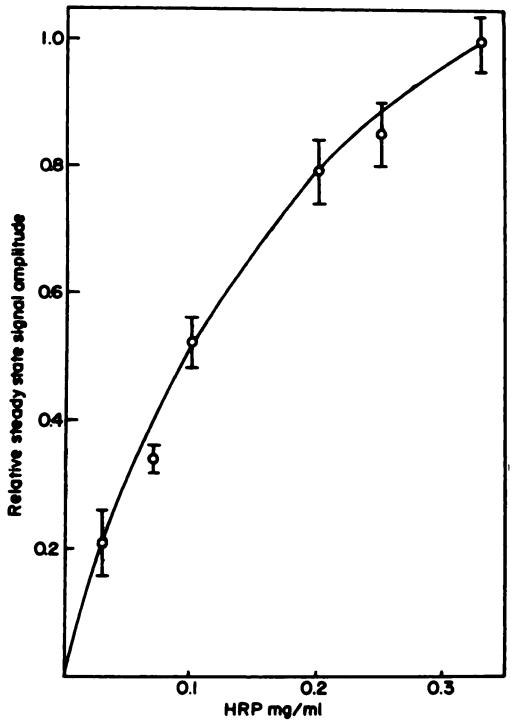

FIG. 2. *Plot of relative steady-state signal intensity versus Type VI HRP concentration (1 mg = 330 units)*

The intensity resulting from reaction of (bi)sulfite with H_2O_2 was subtracted from the intensity observed in the full system. The H_2O_2 concentration was 10 μM and the Na_2SO_3 concentration was 10 mM in boric acid/sodium borate buffer, pH 8.6. The amplitude was measured by fixing the magnetic field at the top of the $\dot{S}O_3^-$ peak. A baseline was obtained with buffer in the flat cell. An incubation was then aspirated into the flat cell without removing the cell from the magnetic field. The amplitude above baseline was measured when a steady state was reached (within 2 min). Modulation amplitude 0.66 G, 20 mW power.

The steady-state concentration of $\dot{S}O_3^-$ in Fig. 1A was 1×10^{-8} M. This concentration was determined by comparing the $\dot{S}O_3^-$ signal intensity to the $\dot{S}O_2^-$ signal intensity obtained from a known concentration of sodium dithionite in buffer [$S_2O_4^{-2} \rightleftarrows 2\dot{S}O_2^-$, $K_{eq} = 1.4 \times 10^{-9}$ M (23).] An $\dot{S}O_3^-$ lifetime of 0.1 sec was determined from the $\dot{S}O_3^-$ concentration and the rate constant for $\dot{S}O_3^-$ decay, $2k = 1.1 \times 10^9$ M^{-1} sec^{-1} (18).

Direct detection of the $\dot{S}O_3^-$ radical required anaerobiosis, implying that the radical reacts with oxygen, as has been proposed (4, 5, 7, 24).

$$\dot{S}O_3^- + O_2 \rightarrow SO_3 + \dot{O}_2^- \quad (5)$$

$$\text{(Bi)sulfite} + \dot{O}_2^- + (1 \text{ or } 2)H^+ \rightarrow \dot{S}O_3^- + H_2O_2 \quad (6)$$

$$\dot{S}O_3^- + O_2 \rightarrow \dot{S}O_5^- \quad (7)$$

$$\text{(Bi)sulfite} + \dot{S}O_5^- \rightarrow \dot{S}O_3^- + SO_5^{2-} + (0 \text{ or } 1)H^+ \quad (8)$$

Apparently, the reactions consuming $\dot{S}O_3^-$ (Eqs. 5 and/or 7) are much faster than the reactions that regenerate the $\dot{S}O_3^-$ radical (Eqs. 6 and/or 8), otherwise $\dot{S}O_3^-$ would be observable under aerobic conditions.

In order to show that the $\dot{S}O_3^-$ formation was enzymatic, the dependence of the steady-state $\dot{S}O_3^-$ signal intensity on horseradish peroxidase concentration was determined (Fig. 2). In the absence of linewidth changes (which were not observed), the ESR signal amplitude is proportional to the $\dot{S}O_3^-$ concentration. The steady-state $\dot{S}O_3^-$ concentration increased with increasing enzyme concentration, but the signal-to-noise ratio was too low to determine further the relationship of the $\dot{S}O_3^-$ concentration to the enzyme concentration.

Equations 2 and 3 were predicted by Araiso *et al.* (17) to be major pathways in the pH range from 5 to 8 when acidic isoenzymes are used, but at pH values greater than

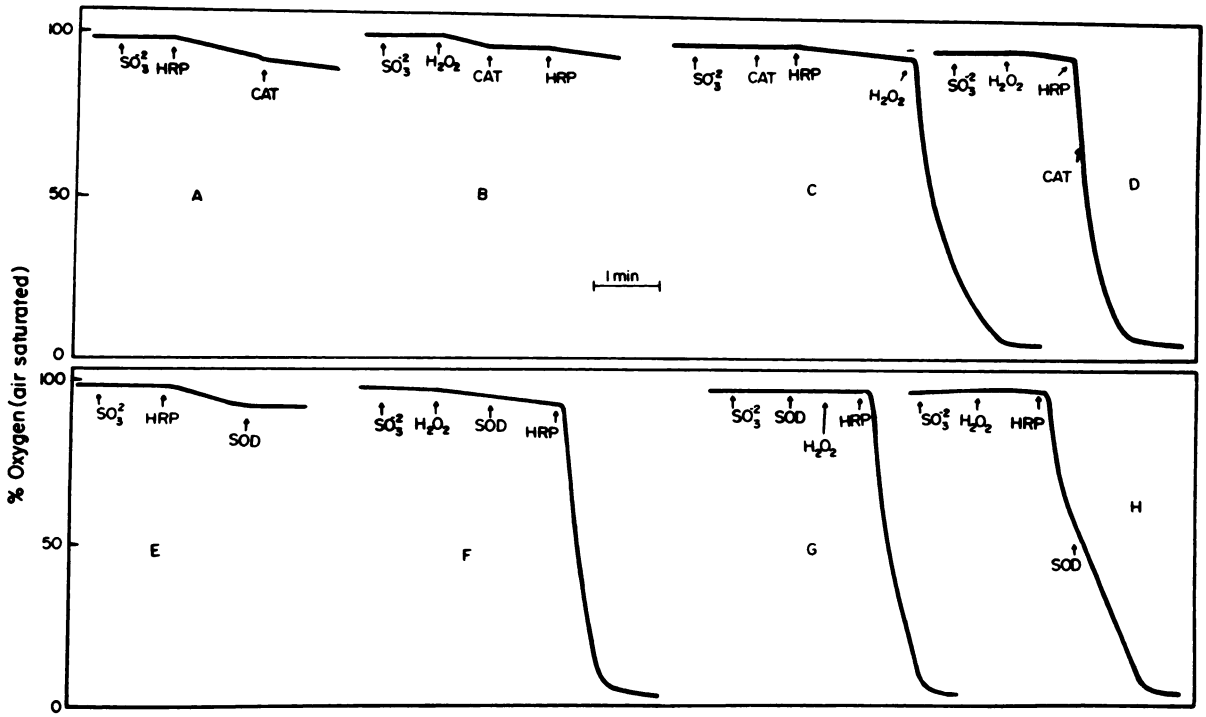

FIG. 3. *Oxygen uptake curves for oxidation of sulfite by the HRP/H_2O_2 system at 25°*

All solutions were 1 mM in DETAPAC and were in boric acid/sodium borate buffer, pH 8.6. Sulfite concentration, 10 mM; HRP Type VI, 0.25 mg/ml (83 units/ml); H_2O_2, 10 μM; superoxide dismutase, 40 μg/ml; catalase, 30,000 units/ml.

7.7 when the basic isoenzymes of horseradish peroxidase are present. In fact, we observed this behavior. At pH less than 4.5, for the acidic isoenzyme, and pH less than 7.7, for the basic isoenzyme, no $\dot{S}O_3^-$ is observed; this supports the predominance of the one-step, two-electron transfer from (bi)sulfite to horseradish peroxidase Compound I with little Compound II formation under these pH conditions (17). Presumably this reaction forms sulfate as the first enzyme-free intermediate

$$\text{HRP-Compound I} + \text{(bi)sulfite} \rightarrow \text{HRP} + SO_4^{2-} \qquad (9)$$

and may be classified as a hydroxylation of bisulfite (25).

In order to relate these ESR investigations to the earlier investigations of the horseradish peroxidase/H_2O_2/(bi)sulfite system, we have investigated the consumption of oxygen by this system. Figure 3 shows the oxygen uptake at pH 8.6 resulting from the basic isoenzyme system without the complication of (bi)sulfite autoxidation, i.e., all incubations contained 1 mM DETAPAC. As can be seen, the addition of either H_2O_2 or horseradish peroxidase increases oxygen uptake. These reactions are catalase-sensitive (over a range of catalase concentration from 3,000 to 30,000 units/ml), indicating involvement of H_2O_2 (Eq. 4), and superoxide dismutase-sensitive (inhibition could be observed with enzyme concentrations as low as 4 μg/ml), indicating involvement of superoxide (Eqs. 5 and 6).

The addition of both H_2O_2 and horseradish peroxidase (order does not matter) greatly stimulates oxygen uptake (Fig. 3C and D). Surprisingly, this reaction was neither catalase- nor superoxide dismutase-sensitive. The insensitivity to superoxide dismutase probably indicates that this pathway does not involve a superoxide chain reaction. The absence of a catalase effect is surprising. The oxygen in these solutions is in 26-fold excess over the added hydrogen peroxide (260 μM versus 10 μM), which implies that some type of chain reaction (either enzymatic or non-enzymatic) is involved. Hydrogen peroxide reacts non-enzymatically with (bi)sulfite (Fig. 3B), and therefore hydrogen peroxide is not expected to accumulate in (bi)sulfite-containing solutions. If hydrogen peroxide had accumulated, catalase would cause an increase in the oxygen concentration by disproportionating hydrogen peroxide. This type of oxygen recovery was not observed, either during or after the consumption of oxygen by (bi)sulfite solutions. The catalase is active because, in the absence of horseradish peroxidase, it destroys hydrogen peroxide and thereby prevents subsequently added horseradish peroxidase from catalyzing oxygen consumption at a rapid rate (Fig. 3B). However, under these conditions, horseradish peroxidase successfully competes with catalase if both are present when hydrogen peroxide is added. At one-fifth the concentration of horseradish peroxidase, catalase partially inhibits this slower oxygen consumption (Fig. 4). Inhibition occurs if catalase is added either before (A) or after (B) hydrogen peroxide. The inhibition is still not complete; apparently, even in the presence of catalase, enough peroxide, perhaps O_3SOOH, reacts with the horseradish peroxidase to sustain the oxidation of (bi)sulfite. Superoxide dismutase (40 μg/ml) had no effect on the oxygen consumption shown in Fig. 4, either in the absence or presence of catalase.

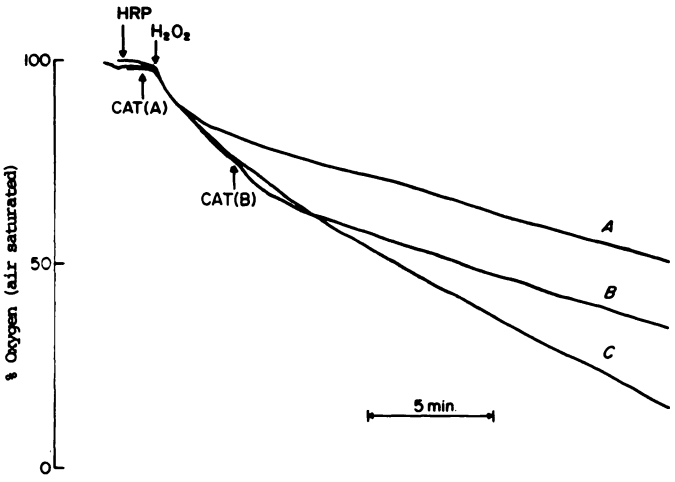

FIG. 4. *Oxygen uptake curves identical with those described in Fig. 3, except that the horseradish peroxidase concentration was* 0.05 *mg/ml*

The oxidation of indole-3-acetic acid by horseradish peroxidase is not inhibited by a catalytic amount of catalase. This result is best explained by the reaction of the indole-3-acetic acid-derived free radical with oxygen to form a hydroperoxide species, which reacts with horseradish peroxidase to form Compound I (26). A similar explanation may apply to the relative insensitivity of (bi)sulfite oxidation to catalase.

Araiso *et al.* (17) have reported rate constants for the one- and two-electron reductions of Compound I of the acidic (peroxidase C) and the basic (peroxidase A) isoenzymes by (bi)sulfite as a function of pH. Although the reduction of Compound I by (bi)sulfite is probably not the rate-limiting step in the consumption of oxygen by these systems, we have investigated its pH dependence in the presence and absence of these isoenzymes. Even in the presence of 1 mM DETAPAC, the autoxidation of (bi)sulfite occurs at pH values below 7.7 (Fig. 5). The rate of oxygen consumption is greatest at about pH 6. A previous investigation of the ferric iron catalysis of

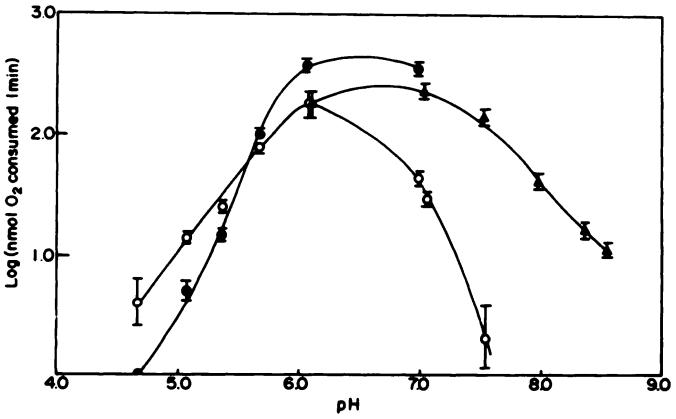

FIG. 5. *pH Profile of oxygen uptake for enzymatic and non-enzymatic sulfite oxidation at 25°*

Final solutions were 3.33 mM in sulfite and 10 μM in H_2O_2, and contained 20 purpurogallin units of HRP activity in a total volume of 3 ml. For pH < 7.0, sodium acetate/acetic acid buffer was used, and for pH > 7.0, Tris-HCl buffer was used. ○, Autoxidation; △, basic isoenzyme; ●, acidic isoenzyme. Type VI horseradish peroxidase, which contains two basic isoenzymes, followed the same course as the basic isoenzyme.

(bi)sulfite oxidation found a pH optimum at 4 (27). At pH values less than 5.5 the acidic isoenzyme inhibits the autoxidation of (bi)sulfite (Fig. 4). Concomitantly, the two-electron reduction of Compound I (Eq. 9) becomes more important relative to one-electron transfer (17). This reaction consumes H_2O_2 via Eqs. 1 and 9 without forming $\dot{S}O_3^-$ and, therefore, has the same effect as catalase (i.e., the H_2O_2-dependent oxygen consumption is inhibited). Above pH 5.5 the formation of sulfate by the acidic isoenzyme is almost entirely the result of $\dot{S}O_3^-$ formation (17), and thus peroxidase stimulates the autoxidation via Eqs. 5, 6, 7, and/or 8. The results are similar for the basic isoenzyme, with a marked stimulation of oxygen consumption at higher pH values, although the pH at which this enzyme stimulates oxygen consumption cannot be easily understood. The decomposition of the Compound II/sulfite complex, or the reduction of Compound II, is probably rate-limiting in the turnover of horseradish peroxidase (17); therefore a good correlation between one- and two-electron reduction of Compound I and oxygen consumption cannot be expected.

DISCUSSION

In this work, the direct observation of the $\dot{S}O_3^-$ radical in the (bi)sulfite/horseradish peroxidase/H_2O_2 system is reported, providing proof of its role in the mechanism of (bi)sulfite oxidation via this system. Previous work on the oxidation of (bi)sulfite by the horseradish peroxidase/H_2O_2 system provided only indirect evidence for the (bi)sulfite-derived free radical, primarily Compound II formation (17). Although Araiso *et al.* (17) wrote the proposed radical as the protonated form, $H\dot{S}O_3$, the ESR spectrum does not show any resolved proton hyperfine splitting, which would be expected for the species $H\dot{S}O_3$. This result implies the formation of the radical anion $\dot{S}O_3^-$. Clearly, Compound II formation provides no information on the structure of free radical metabolites, other than to necessitate that they be a one-election oxidation product of the substrate.

ESR studies under anaerobic conditions and oxygen consumption studies are in agreement that radical formation is more important at basic pH values, and that the pH dependence varies with the particular isoenzyme. The pH dependence of the steady-state $\dot{S}O_3^-$ concentration reflects the rate of radical formation by Compounds I and II, and not the rate of radical decay, which is nearly pH-independent (18). Since the ESR signal was not observed under aerobic conditions, the reactions of Eq. 5 and/or Eq. 7 must occur quite rapidly. When all of the oxygen was consumed, the $\dot{S}O_3^-$ ESR signal was observed, implying that the decay processes (18)

$$2\dot{S}O_3^- \rightarrow SO_3 + SO_3^{2-} \quad (10)$$

$$SO_3 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (11)$$

and

$$2\dot{S}O_3^- \rightarrow S_2O_6^{2-} \quad (12)$$

were not as rapid as the reactions of Eq. 5 and/or Eq. 7. The final step in the sequence is the reaction of SO_3 with water to form the sulfate dianion (Eq. 11).

Once the $\dot{S}O_3^-$ radicals are formed, they can react with oxygen (Eq. 5, 6, and 7), or the radicals can undergo disproportionation (Eq. 10) and/or dimerization (Eq. 12). The total insensitivity of the oxygen uptake experiment to superoxide dismutase implies that, once the reaction is initiated, superoxide is not involved in chain propagation. Apparently, the chain reaction of Eqs. 5 and 6 is unimportant relative to the enzymatic reactions of Eqs. 2 and 3 in the formation of $\dot{S}O_3^-$ and the subsequent oxygen consumption.

Equation 4, the reaction of hydrogen peroxide with (bi)sulfite to give the $\dot{S}O_3^-$ anion radical, is of particular interest, because it also forms the hydroxyl radical. This reaction, like autoxidation, is probably catalyzed by trace transition metals that, in their reduced form, reduce hydrogen peroxide to form the hydroxyl radical. This would be completely analogous to the recently reported iron-EDTA catalysis of hydroxyl radical formation by ascorbate and hydrogen peroxide (28).

Since peroxidase enzymes and H_2O_2 exist in mammalian systems, this may be a possible pathway for the conversion of (bi)sulfite to sulfate for elimination. The horseradish peroxide/H_2O_2 system often catalyzes reactions which are identical with those catalyzed by myeloperoxidase, lactoperoxidase (29), and prostaglandin synthetase/hydroperoxidase (30). This free radical metabolism of sulfite is in contrast to the oxidation of sulfite by sulfite oxidase, which is thought to detoxify sulfite by forming sulfate, without forming significant amounts of free radicals (31). (Bi)sulfite autoxidation is not thought to occur *in vivo*, and consequently the free-radical reactions of (bi)sulfite have a highly speculative role in (bi)sulfite toxicity (32). The peroxidase-catalyzed formation of the sulfur trioxide anion radical opens the possibility of the free-radical metabolism of (bi)sulfite *in vivo*.

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