Copper, Zinc Superoxide Dismutase and H$_2$O$_2$

EFFECTS OF BICARBONATE ON INACTIVATION AND OXIDATIONS OF NADPH AND URATE, AND ON CONSUMPTION of H$_2$O$_2$*

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Copper, zinc superoxide dismutase (Cu,Zn-SOD) catalyzes the HCO$_3^-$-dependent oxidation of diverse substrates. The mechanism of these oxidations involves the generation of a strong oxidant, derived from H$_2$O$_2$, at the active site copper. This bound oxidant then oxidizes HCO$_3^-$ to a strong and diffusible oxidant, presumably the carbonate anion radical that leaves the active site and then oxidizes the diverse substrates. Cu,Zn-SOD is also subject to inactivation by H$_2$O$_2$. It is now demonstrated that the rates of HCO$_3^-$-dependent oxidations of NADPH and urate exceed the rate of inactivation of the enzyme by ~100-fold. Cu,Zn-SOD is also seen to catalyze a HCO$_3^-$-dependent consumption of the H$_2$O$_2$ and that HCO$_3^-$ does not protect Cu,Zn-SOD against inactivation by H$_2$O$_2$. A scheme of reactions is offered in explanation of these observations.

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1 The abbreviation used is: SOD(s), superoxide dismutase(s).

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All superoxide dismutases (SODs)$^1$ catalyze the conversion of O$_2^*$ into H$_2$O$_2$ plus O$_2$ and do so with great efficiency. The Cu,Zn-SOD is also able to catalyze other processes, albeit at lower rates, including: the HCO$_3^-$-dependent oxidation of diverse substrates by H$_2$O$_2$ (1–5); the reduction of O$_2^*$ by substrates other than O$_2^*$ (6), referred to as superoxide reductase activity; and the elimination of NO$^*$ both aerobically and anaerobically (7, 8). The inactivation of Cu,Zn-SOD by H$_2$O$_2$ and its ability to act as a nonspecific peroxidase were described (1, 9) long before the dependence of the latter activity on HCO$_3^-$ was appreciated (2–5).

The relevant literature has been marred by controversy. Thus, the production of HO$^*$ as the oxidant responsible for both inactivation by H$_2$O$_2$ and for the peroxidase activity has been claimed (5) and denied (3, 4, 10). The bicarbonate effect has been attributed to facilitation of the binding of H$_2$O$_2$ (2), and that explanation has been called into question (3, 4). Bicarbonate has been reported to protect (3, 11) and not to protect (4) against the inactivation of the enzyme by H$_2$O$_2$. Finally, the rate constant for reaction leading to the formation of the copper-bound oxidant was estimated to be only 0.1 M$^{-1}$ s$^{-1}$ at pH 7.4 (12), which is not fast enough to account for the rapid HCO$_3^-$-dependent oxidations (2, 4).

Clearly the mechanism of the interaction of H$_2$O$_2$ with the Cu,Zn-SOD and the effects of HCO$_3^-$ require further clarification. We now present data and calculations derived therefrom, which shed some light on these reactions. The following scheme of reactions reflect current knowledge. Elaborations, to be presented under “Discussion” will lead to a scheme more in tune with observed reactions and their rates.

\[
E\rightarrow Cu(II) + H_2O_2 \rightleftharpoons E\rightarrow Cu(I) + O_2 + 2H^+
\]

REACTION a

\[
E\rightarrow Cu(I) + H_2O_2 \rightarrow E\rightarrow Cu(II)OH + OH^-
\]

REACTION b

\[
E\rightarrow Cu(II) + O_2 \rightarrow E\rightarrow Cu(I) + O_2
\]

REACTION c

\[
E\rightarrow Cu(II)OH + His \rightarrow E\rightarrow Cu(II) + OH^- + His^+
\]

REACTION d

\[
E\rightarrow Cu(II)OH + HCO_3^- \rightarrow E\rightarrow Cu(II) + H_2O + CO_3^-
\]

REACTION e

\[
CO_3^- + SH_2 \rightarrow HCO_3^- + SH
\]

REACTION f

\[
CO_3^- + His + H^+ \rightarrow HCO_3^- + His^+
\]

REACTION g

\[
Cu(II)OH + urate \rightarrow Cu(II) + OH^- + urate
\]

REACTION h

In this scheme SH$_2$ denotes a substrate such as NADPH, and His denotes a histidine residue in the ligand field of the copper. This scheme is very similar to the one that we arrived at previously (4). In Reaction a the enzyme is univalently reduced by H$_2$O$_2$, while in Reaction b the bound oxidant is generated. We consider the oxidant to be bound rather than diffusible because HO$^*$ scavengers, such as ethanol, do not protect (1) and because the oxidation of large substrates depends upon the mediation of bicarbonate (2–5). The bound oxidant can inactivate the enzyme by oxidation of a histidine residue (Reaction d) or, it can oxidize HCO$_3^-$ to the powerful oxidant CO$_3^-$ which then leaves the active site and oxidizes substrates in free solution (Reaction f) or alternately stays in the active site to oxidize a histidine residue (Reaction g). Reaction g was proposed because HCO$_3^-$ competes with histidine (Reactions d and e) and should thus protect, which it does not (4). Urate does protect the enzyme, hence Reaction h.
Materials and Methods

NADPH, horseradish peroxidase, and diansidine were from Sigma. The diansidine was twice recrystallized from ethanol. Uric acid and NaHCO₃ were from Fisher, H₂O₂ was from Mallinkrodt, and Cu,Zn-SOD was from Chemie Grunenthal. All experiments were performed in 100 mM sodium phosphate at pH 7.4 and at 22–23 °C. When NaHCO₃ was added the buffer was readjusted to pH 7.4 with H₂SO₄ as needed. Initial rates of oxidation of NADPH were followed at 340 nm and of urate at 295 nm. The usual concentrations of SOD and H₂O₂ were 0.2 mg/ml and 10 mM, respectively. When [NADPH] or [urate] were varied the HCO₃⁻ was held constant at 50 mM in the case of NADPH and at 150 mM in the case of urate. When [HCO₃⁻] was varied, urate and NADPH were held constant at 60 and 100 μM, respectively. When urate oxidation was examined in the absence of HCO₃⁻, the [SOD] was raised to 0.8 mg/ml. The results were then corrected to the usual condition by dividing by four.

Inactivation of SOD by H₂O₂ was followed by removing samples at intervals and assaying remaining SOD activity by the xanthine oxidase/xanthine method (13). Consumption of H₂O₂ by Cu,Zn-SOD was followed by removing samples at intervals, assaying for residual [H₂O₂] by 25-fold dilution into buffered (pH 7.4) solutions of 0.3 mM dianisidine plus 40 μg/ml horseradish peroxidase, and measuring absorbance at 460 nm. The reduction of 5.0 mg/ml Cu(II),Zn SOD by 2 mM H₂O₂ was followed at 680 nm.

Results

HCO₃⁻-dependent Oxidation of NADPH and Urate—Were the processes presented by Reactions a–h correct we would expect the bound oxidant produced by Reaction b to inactivate the enzyme by Reaction d or, in the presence of HCO₃⁻, to oxidize extraneous substrates through the mediation of CO₃²⁻. The rate of inactivation should in any case equal the maximal rate of oxidation of either NADPH or urate. We will now show that the rate of inactivation is 100-fold slower than the maximal rate of oxidation of either NADPH or urate.

Figs. 1 and 2 present the rates of oxidation of urate and of NADPH as a function of HCO₃⁻. Because the effect of HCO₃⁻ approaches a limit we can use reciprocal coordinates to obtain the maximal rate of oxidation of infinite HCO₃⁻, which will be referred to as V_max, and the concentration of HCO₃⁻ that caused half that maximal rate, which will be referred to as K_m. Fig. 3 shows the data for NADPH oxidation plotted on reciprocal coordinates, and Table I, lines 1 and 2 present K_m and V_max for the HCO₃⁻ effect on both urate and NADPH oxidation obtained at the indicated concentrations of H₂O₂ and Cu,Zn-SOD. Lines 3 and 4 present K_m and V_max values for NADPH and urate as the variable substrates at fixed [HCO₃⁻].

The K_m for HCO₃⁻ was approximately equal for both NADPH and urate oxidation. This is expected because both oxidations depend upon the CO₃²⁻ leaving the active site. Were NADPH and urate comparable processes we would expect that V_max should also be the same for both oxidations. However, V_max for NADPH oxidation was 5× greater than V_max for urate oxidation. This difference may be partially artifactual. Thus oxidation of NAPDH by CO₃⁻ would be followed by the auto-oxidation of NADP⁺ to NADPH⁺ + O₂ with loss of 340 nm of absorbance. In contrast the oxidation of urate can involve several intermediates, such as allantoin and allantoic acid (14), and some of those may absorb at 295 nm, as does urate, and may also consume CO₂. It is also possible that urate, being an anion, may be less reactive with CO₃²⁻ than is the uncharged dihydropyridine moiety of NAPDH.

When [HCO₃⁻] was constant at 50 mM and NADPH was varied the K_m for NADPH was less than 17 μM. Hence 100 μM NADPH will scavenge essentially all the CO₃²⁻ leaving the active site, and the V_max for NADPH oxidation, 20 μM/min, must approximate the rate of production of CO₃²⁻ K_m for urate was also low (~7.5 μM). The V_max obtained by extrapolating to infinite HCO₃⁻ and at 100 μM NADPH was 50 μM/min, which must then approximate the rate of production of the bound oxidant (Reaction b).

Urata oxidation in the absence of HCO₃⁻ was so slow that it was advisable to raise [SOD] to achieve a measurable rate. This
Effects of \( \text{HCO}_3^- \) on \( \text{H}_2\text{O}_2/\text{Cu,Zn-SOD} \) Interactions

TABLE I

| Substrate       | Means of measurement | What is varied | \( K_{\text{max}} \) | \( V_{\text{max}} \) |
|-----------------|----------------------|----------------|---------------------|---------------------|
| Bicarbonate     | NADPH-oxidation      | \( \text{HCO}_3^- \) (10–500 mM) | \( \sim 100 \text{ mM} \) | 50 \( \mu\text{m} / \text{min} \) |
| Bicarbonate     | Urate-oxidation      | \( \text{HCO}_3^- \) (10–500 mM) | \( \sim 80 \text{ mM} \) | 10 \( \mu\text{m} / \text{min} \) |
| Bicarbonate     | NADPH-oxidation      | \( \text{NADPH} \) (12.5–100 \( \mu\text{m} \)) | \( <17 \mu\text{m} \) | 20 \( \mu\text{m} / \text{min} \) |
| Bicarbonate     | Urate-oxidation      | Urate (7.5–80 \( \mu\text{m} \)) | \( <7.5 \mu\text{m} \) | 9.0 \( \mu\text{m} / \text{min} \) |

In all cases [SOD] was at 0.2 mg/ml and [\( \text{H}_2\text{O}_2 \)] at 10 mM. When [NADPH] was varied [\( \text{HCO}_3^- \)] was at 50 mM, but when urate was varied [\( \text{HCO}_3^- \)] was at 150 mM. When [\( \text{HCO}_3^- \)] was varied [NADPH] was at 100 \( \mu\text{m} \) and [urate] at 60 \( \mu\text{m} \). \( K_{\text{max}} \) and \( V_{\text{max}} \) for urate oxidation in the absence of \( \text{HCO}_3^- \) were \( \sim 25 \mu\text{m} \) and 0.7 \( \mu\text{m}/\text{min} \), respectively. Urate oxidations in this case were performed at 10 mM \( \text{H}_2\text{O}_2 \) but at 0.8 mg/ml SOD. Hence the observed \( V_{\text{max}} \) should be divided by four for comparison with the other \( V_{\text{max}} \) in the table, i.e. \( 0.7/4 = 0.18 \mu\text{m} / \text{min} \).

Direct oxidation of urate should reflect the rate of Reaction i. The \( V_{\text{max}} \) urate of 0.7 \( \mu\text{m} / \text{min} \), which would be 0.18 \( \mu\text{m} / \text{min} \) at 0.2 mg/ml SOD, is more than 50 \( \times \) smaller than the rate in the presence of saturating \( \text{HCO}_3^- \). Urate is thus a poorer substrate for the bound oxidant than is \( \text{HCO}_3^- \), yet urate protects while \( \text{HCO}_3^- \) appears not to do so (4). How a molecule as large as urate accesses the active site and protects against inactivation by the bound oxidant (Reaction d) remains a puzzle.

Does \( \text{HCO}_3^- \) Protect Cu,Zn-SOD against Inactivation by \( \text{H}_2\text{O}_2 \)?—Fig. 4 presents a semilog plot of residual SOD activity during exposure to excess \( \text{H}_2\text{O}_2 \). Rather than protect, as has been reported by others (3, 11), \( \text{HCO}_3^- \) modestly increased the rate of inactivation. We previously reported (4) that 10 mM \( \text{HCO}_3^- \) did not protect in accordance with the results in Fig. 4 where higher concentrations were needed to show a measurable acceleration. The rate of inactivation from Fig. 4 was \( \sim 100 \) -fold slower than the \( V_{\text{max}} \) for the \( \text{HCO}_3^- \)-dependent oxidation of NADPH. Thus the \( V_{\text{max}} \) for NADPH oxidation from Table I was 50 \( \mu\text{m} / \text{min} \), while the inactivation of the Cu,Zn-SOD by 10 mM \( \text{H}_2\text{O}_2 \), calculated from Fig. 4 on the basis of a subunit mass of 16,000 Da, was 0.5 \( \mu\text{m} / \text{min} \). This discrepancy indicates that only a small fraction (1%) of the bound oxidant potentially produced by Reaction b is available for Reaction d. An explanation will be presented under “Discussion.”

\( \text{HCO}_3^- \)-dependent Consumption of \( \text{H}_2\text{O}_2 \)—Fig. 5 demonstrates that \( \text{HCO}_3^- \) greatly increases the consumption of \( \text{H}_2\text{O}_2 \) by Cu,Zn-SOD. The effect of \( \text{HCO}_3^- \) was saturable and the \( V_{\text{max}} \) for this \( \text{H}_2\text{O}_2 \) consumption was similar to that for NADPH oxidation. If the \( \text{H}_2\text{O}_2 \) reacted rapidly with the bound oxidant its rate of consumption would be high in the absence of \( \text{HCO}_3^- \), which is not the case. But we know that \( \text{H}_2\text{O}_2 \) rapidly reduces the Cu(II) enzyme (Reaction a). Hence if \( \text{HCO}_3^- \) has the effect of converting the bound oxidant back to the Cu(II) enzyme by Reaction e that alone would explain the augmentation of \( \text{H}_2\text{O}_2 \) consumption by \( \text{HCO}_3^- \); this will be made clear later in the text. Is it also plausible that \( \text{CO}_2 \) can oxidize \( \text{H}_2\text{O}_2 \) as in Reaction i?

\[
\text{CO}_2 + \text{H}_2\text{O}_2 \rightarrow \text{HCO}_3^- + \text{H}^+ + \text{O}_2
\]

and thus account for a significant portion of \( \text{HCO}_3^- \)-augmented \( \text{H}_2\text{O}_2 \) consumption. It is immediately apparent that this is unlikely. Thus half-maximal NADPH oxidation was seen at \( \sim 10 \mu\text{m} \) NADPH when \( \text{H}_2\text{O}_2 \) was at 10 mM. A thousand-fold excess of \( \text{H}_2\text{O}_2 \) thus could not compete with NADPH for the flux of \( \text{CO}_2 \). It follows that the rate constant for Reaction i must be too low to contribute significantly to the observed \( \text{H}_2\text{O}_2 \) consumption.

Rate of Reduction of Cu(II)-Zn-SOD by \( \text{H}_2\text{O}_2 \)—SOD (5 mg/ml) was treated with 2 mM \( \text{H}_2\text{O}_2 \) at pH 7.4, and in the absence of \( \text{HCO}_3^- \) as shown in Fig. 6 the rate of reduction, followed in terms of the bleaching of absorbance at 680 nm, was very rapid. Thus half (78 \( \mu\text{m} \)) of the SOD was bleached within 10–15 s. Because the rate was declining rapidly within this time inter-
Effects of HCO\textsubscript{3} on H\textsubscript{2}O\textsubscript{2}/Cu\textsubscript{2}Zn-SOD Interactions

**DISCUSSION**

Because HCO\textsubscript{3} does not much change the rate of inactivation of Cu,Zn-SOD by H\textsubscript{2}O\textsubscript{2}, we can use the reaction scheme of (3, 11) to reexamine the role of carbonate in protecting Cu,Zn-SOD from H\textsubscript{2}O\textsubscript{2}.

REACTION j

\[
\text{Cu(II)OH} \rightarrow \text{Cu(III)} + \text{OH}^-.
\]

We could further suppose that Cu(III) was able to attack a ligandizing histidine while Cu(II)OH could react with HCO\textsubscript{3} but not with the histidine. The Cu(III) could also be responsible for the slow HCO\textsubscript{3}-independent oxidations of urate, formate, and H\textsubscript{2}O\textsubscript{2}. This could explain the slowness of those reactions as well as the protective effects of urate and formate (9, 12).

REACTION k

\[
\text{E—Cu(II)H}_2\text{O}_2 \rightarrow \text{Cu(II)OH} + \text{OH}^-.
\]

In this case the rapid HCO\textsubscript{3}-dependent oxidations follow the oxidation of HCO\textsubscript{3} by E-Cu(II)H\textsubscript{2}O\textsubscript{2}, while the slow HCO\textsubscript{3}-independent inactivation and oxidations depend on E-Cu(II)-OH produced by the slow Reaction I. In this case the urate protects by reacting with E-Cu(II)OH in competition with the histidine residue, but is not in competition with HCO\textsubscript{3} for E-Cu(II)H\textsubscript{2}O\textsubscript{2}, which rapidly decomposes to E-Cu(II) + H\textsubscript{2}O\textsubscript{2}.

**Rate Constants**—Having measured rates at known concentrations of reactants, we can now calculate apparent second order rate constants. Thus: \[ V = k [R_1] [R_2] \] In this case \( R_1 \) and \( R_2 \) denote reactants such as Cu(II)Zn-SOD, Cu(II)Zn-SOD, and H\textsubscript{2}O\textsubscript{2}. Table II presents the results of such calculations. For the case of HCO\textsubscript{3}-dependent H\textsubscript{2}O\textsubscript{2} consumption we estimated ini-
tial rates by correcting for the concurrent inactivation of the SOD during the period of observation and for the consumption of H$_2$O$_2$ during that period. None of these corrections introduced more than a factor of 2.0–2.5 into the calculated rate constant. The rate of production of the bound oxidant Cu(II)OH ($V_{b}$) must, in the steady state, equal its rate of consumption by the reverse reaction ($V_{b-}$) and by the oxidation of an active site histidine ($V_{d}$). Hence, $V_{b} = V_{b-} + V_{d}$ and then $V_{b-} = V_{b} - V_{d}$.

If Reaction $k$ actually occurs and not Reaction $b_{1}$, $V_{l} = V_{d}$ and then the above equation should be replaced by $V_{b-} = V_{b} - V_{l}$. As previously stated the rate of production of the bound oxidant can be equated to the maximal rate of HCO$_3$-$\text{dependent oxidation of NADPH}$ or consumption of H$_2$O$_2$. In calculating $V_{b}$ and $k_{b}$ we take the [Cu(I),Zn-SOD] from the total concentration of SOD even though some fraction of the SOD must remain in the Cu(II) form even in the presence of H$_2$O$_2$. Hence our estimate of $k_{b}$ is a minimal figure. Nevertheless we find $k_{b}$ to be ~100-fold higher than the value reported by Cabelli et al. (12). In their system formate was oxidized by the bound oxidant and the resultant CO$_2$ reduced nitroblue tetrazolium. This is quite analogous to our use of HCO$_3$ followed by oxidation of NADPH by CO$_2$. A possible explanation for the very low rate of Reaction $b_{+}$ found by Cabelli et al. (12) is that CO$_2$ did not predominantly escape the active site but rather reduced the E–Cu(II) to E–Cu(I). This reaction is known to occur (16). Our high estimate for the rate of Reaction $b_{+}$ accounts for the rapid HCO$_3$-dependent oxidations reported by Hodgson and Fridovich (1), Sankarapandi and Zweier (2), and in the present work. It has become obvious from the results and discussion presented above that in the absence of HCO$_3$ no significant oxidant leaves the active site of Cu,Zn-SOD in the presence of H$_2$O$_2$. Hence the assertion that this enzyme catalyzes the production of HO$^-$ from H$_2$O$_2$ can be laid to rest.

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