Nitroxyl (NO•) may be produced by nitric-oxide synthase and by the reduction of NO by reduced Cu,Zn-SOD. The ability of NO• to cause oxidations and of SOD to inhibit such oxidations was therefore explored. The decomposition of Angeli’s salt (AS) produces NO• and that in turn caused the aerobic oxidation of NADPH, directly or indirectly. O2•− was produced concomitant with the aerobic oxidation of NADPH by AS, as evidenced by the SOD-inhibitable reduction of cytochrome c. Both Cu,Zn-SOD and Mn-SOD inhibited the aerobic oxidation of NADPH by AS, but the amounts required were −100-fold greater than those needed to inhibit the reduction of cytochrome c. This inhibition was not due to a nonspecific protein effect or to an effect of those large amounts of the SODs on the rate of decomposition of AS. NO•− caused the reduction of the Cu(II) of Cu,Zn-SOD, and in the presence of O2•− SOD could oxidize the activity of NO•− to NO. The reverse reaction, i.e. the reduction of NO to NO•− by Cu(II),Zn-SOD, followed by the reaction of NO•− with O2 would yield ONOO− and that could explain the oxidation of dichlorofluorescein (DCF) by Cu(II),Zn-SOD plus NO. Cu,Zn-SOD plus H2O2 caused the HCO3−-dependent oxidation of DCF, casting double on the validity of using DCF oxidation as a reliable measure of intracellular H2O2 production.

SODs protect against the deleterious actions of O2•− by catalyzing its dismutation (1). However, additional properties have been ascribed to these enzymes, including peroxidase (2–4), superoxide reductase (5), superoxide oxidase (5), and reversible NO/NO•− oxidoreductase (6–9) activities. The biological significance of these latter activities remain to be solidified, but interest in them is increased by the finding that point mutations in Cu,Zn-SOD, most of which do not effect the SOD but have special significance in view of reports that the ALS-associated mutant Cu,Zn-SODs exhibit lowered affinity for Zn(I) (14, 15) and that Cu(II)-SODs, whether mutant or wild type, were toxic to motor neurons (12).

An alternative explanation for the results of Estevez et al. (12) would entail the reduction of NO to NO•− by the Cu(I)-SOD, followed by production of ONOO− from the reaction of NO•− with O2. The ONOO− thus produced would oxidize DCF. That reduced SODs can act as univalent reductants for NO was shown by Murphy and Sies (6) and by Kim et al. (9) and might account for the observations of McBride et al. (16) in which case H2O2 was the reductant of Cu,Zn-SOD, in place of the ascorbate used by Estevez et al. (12).

MATERIALS AND METHODS

Angeli’s Salt (Na2N2O3; AS) from the Cayman Chemical Co. was generously provided by J. S. Stamler. Stock solutions of ~10 or 20 mM were prepared in 10 mM NaOH and were stored at −20 °C until used. The extinction coefficient of AS at 250 nm in the NaOH was taken to be 8,000 M−1 cm−1 (17) and in sodium phosphate buffer, pH 7.4, to be 4,400 M−1 cm−1. NADPH, diethylenetriamine pentaacetic acid (DTPA), and bovine serum albumin were from Sigma; potassium ferricyanide was from J. T. Baker; cytochrome c was from Fluka; catalase was from Boehringer Ingelheim; bovine Cu,Zn-SOD was from Grunnenthal; and recombinant human Mn-SOD was from Biotechnology General. All reactions were performed at 23 °C in 100 mM sodium phosphate, 50 μM DTPA at pH 7.4. The decomposition of AS was followed at 250 nm (17, 18), the oxidation of NADPH at 340 nm (19), the reduction of cytochrome c at 550 nm (20), and the oxidation of DCF at 500 nm (21).

RESULTS

Oxidation of NADPH by NO•− —An alkaline stock solution of AS, when diluted into neutral buffer to 0.175 mM, decomposed at an initial rate of 9 μm/min as shown by line 1 in Fig. 1. When 100 μM NADPH was present it was oxidized by the AS at a rate of 5 μm/min (line 2). Thus the ratio of AS decomposed to NADPH oxidized was almost 2:1, in agreement with earlier reports (22, 23). Since AS decomposition is known to be a source of NO•− (7, 18, 23, 24), it appears that NO•−, or a species derived therefrom, under aerobic conditions, probably ONOO−, oxidizes NADPH. Both Cu,Zn-SOD and Mn-SOD were able to inhibit the oxidation of NADPH by NO•− as shown in Fig. 2 and in agreement with Reif et al. (22). This was not due to an effect
on the rate of decomposition of AS, which was not influenced by Cu,Zn-SOD (not shown). Moreover it was not due to a nonspecific protein effect, since comparable levels of serum albumin caused only a small and transient effect (Fig. 3) that was probably due to the consumption of NO\(_2^-\)/H\(_2\)O\(_2\) by oxidation of albumin cysteine and methionine residues. Thus GSH was also able to inhibit the oxidation of NADPH by NO\(_2^-\)/H\(_2\)O\(_2\) in a way that suggested consumption of the GSH (Fig. 4).

The inhibitory effect of the SODs did not appear to be due to the dismutation of O\(_2^-\), since the levels of SOD needed to cause the inhibitions seen in Fig. 2 are ~100-fold greater than would be required to cause comparable inhibition of the reduction of cytochrome c by O\(_2^-\). An interesting possibility is that NO\(^-\) can reduce the redox-active metals (Cu(II) or Mn(III)) of the SODs and that O\(_2^-\) then reoxidizes these metal centers, thus providing for catalytic consumption of NO\(^-\). The reactions envisioned are as follows.

\[
\text{Me}(n) + \text{NO}^- \rightleftharpoons \text{Me}(n-1) + \text{NO} \quad \text{REACTION 1}
\]

\[
\text{Me}(n-1) + \text{O}_2^- + 2\text{H}^+ \rightleftharpoons \text{Me}(n) + \text{H}_2\text{O}_2 \quad \text{REACTION 2}
\]

Exploration of this possibility required demonstration that NO\(^-\) could reduce the redox-active metals at the active sites of the SODs and that O\(_2^-\) was produced during the oxidation of NADPH by NO\(^-\).

\(\text{NO}^-\) Reduces Cu,Zn-SOD—Addition of AS to 0.2 mM to a buffered solution of 4 mg/ml of Cu,Zn-SOD resulted in bleaching of the enzyme (Fig. 5). That this bleaching was due to reduction of the Cu(II) to Cu(I) was shown by the ability of ferricyanide to restore the lost absorbance. Catalase did not prevent the reduction of Cu,Zn-SOD by NO\(^-\) (not shown). Thus H\(_2\)O\(_2\) was not involved. This attests to the reality of Reaction 1. The less than complete bleaching shown in Fig. 5 probably was
was produced during the oxidation of NADPH by AS. Subsequent to addition of potassium ferrocyanide to 0.5 mM, the spectrum returned to line 2.

due to our stopping the process after 30 min or to limitation in the amount of AS.

$O_2^-$—Production during the Oxidation of NADPH by NO$^-$
When AS (0.175 mM) was oxidizing aerobic 0.1 mM NADPH, in the presence of 10 or 40 mM cytochrome c, reduction of the cytochrome c was seen (Fig. 6), and this could be inhibited by very low levels of Cu,Zn-SOD. It should be noted that the low levels of Cu,Zn-SOD, that were sufficient to compete with cytochrome c for $O_2^-$, were unable to detectably inhibit NADPH oxidation by NO$^-$. Thus $O_2^-$ was produced during the oxidation of NADPH and that $O_2^-$ could drive Reaction 2. A likely source of that $O_2^-$ would be the oxidation of NADPH to NADP$^+$ by NO$^-$, followed by the autoxidation of NADP$^+$ to NADP$^+$. There was no reduction of cytochrome c by aerobic AS in the absence of NADPH.

Oxidation of Dichlorofluorescin—The oxidation of DCF to its fluorescent product has been widely used as a measure of intracellular production of H$_2$O$_2$ (11, 13, 25), and such use of DCF has been criticized (13). Nevertheless increased DCF oxidation as a consequence of the overproduction of normal Cu,Zn-SOD, or of the FALS-associated mutants, thereof, have been so interpreted (11, 25). We now present data that should decrease enthusiasm for this use of DCF. Thus Fig. 7 demonstrates that DCF is not oxidized by H$_2$O$_2$ per se but is oxidized by Cu,Zn-SOD plus H$_2$O$_2$, and that HCO$_3^-$ markedly stimulates this oxidation. HCO$_3^-$-dependent peroxidations catalyzed by Cu,Zn-SOD have been reported previously (3, 26–28) and have been explained on the basis of the oxidation of HCO$_3^-$ to CO$_3^{2-}$ at the active site, followed by diffusion of the carbonate radical into the bulk solvent where it can cause diverse oxidations (3, 28). Thus the data in Fig. 7 indicates that increased oxidation of DCF within cells could signal increased oxidation of HCO$_3^-$ by Cu,Zn-SOD plus H$_2$O$_2$, due to increased Cu,Zn-SOD, or due to increased H$_2$O$_2$, or to increased peroxidase activities of the FALS-associated, or of the Zn-depleted, enzymes.

DCF can also be oxidized by ascorbate + SOD + a source of NO under aerobic conditions (12). Fig. 8 (line 1) demonstrates that 10 μM DCF is oxidized when exposed to 20 μM AS and that 30 μg/ml Cu,Zn-SOD inhibited. Catalase at 280 units/ml had no significant effect, in the presence or absence of SOD. When 10 μM DCF was exposed to 50 μM AS in an argon-purged buffer, a slow and rapidly decreasing rate of DCF oxidation was seen. Subsequent aeration increased this rate dramatically (line 2). Dihydrorhodamine has similarly been seen to be oxidized by AS in an oxygen-dependent manner (18). It appears that NO$^- + O_2$ yields an oxidant capable of rapid oxidation of DCF or DHR.

We assume that this oxidant is ONOO$^-$, or something derived from it. It has been shown that NO$^-$ produced by Cu(I)-Zn-SOD is in the triplet ground state (9), which is known to most readily react with triplet ground state O$_2$ yielding ONOO$^-$ (24, 29). It should be noted that Miranda et al. (18) proposed that the product of the AS-generated NO$^- + O_2$ reaction is somehow different from ONOO$.^-$. That could be an isomeric form of ONOO$^-$ produced by the reaction of singlet NO$^+$ with O$_2$. However, when NO$^-$ is produced biologically, as in the measurements of Estvez et al. (12), it would be triplet NO$^-$, and this would yield ONOO$^-$, per se, when reacting with O$_2$. In any case ONOO$,^-$ or the similar product proposed by Miranda et al. (18), would rapidly oxidize DCF and DHR.

**Fig. 5. Reduction of Cu,Zn-SOD by AS.** The mixture reaction contained 4 mg/ml Cu,Zn-SOD in 100 mM sodium phosphate, 50 μM DTPA, pH 7.4. Line 1, spectrum before adding AS; line 2, immediately after mixing in AS to 0.2 mM; line 3, 5 min after addition of AS; line 4, 30 min after addition of AS. Subsequent to addition of potassium ferrocyanide to 0.5 mM, the spectrum returned to line 2.

**Fig. 6. Production of O$_2^-$ during NADPH oxidation by AS.** Conditions were the same as described in the legend to Fig. 1 with the following additions: line 1, 10 μM cytochrome c, then Cu,Zn-SOD to 1 μg/ml at the first arrow and to 4 μg/ml at the second arrow; line 2: 40 μM cytochrome c, then Cu,Zn-SOD to 1 μg/ml at first arrow and to 4 μg/ml at the second arrow.

**Fig. 7. Bicarbonate-dependent oxidation of dichlorofluorescin by Cu,Zn-SOD + H$_2$O$_2$.** Reaction mixtures contained 100 mM sodium phosphate, 50 μM DTPA, 10 μM DCF, and 2.0 mM H$_2$O$_2$. DCF oxidation was followed at 500 nM. Line 1, Cu,Zn-SOD added to 0.15 mg/ml at the first arrow and sodium bicarbonate to 10 mM at the second arrow. Line 2, all components present except Cu,Zn-SOD, which was added to 0.15 mg/ml at the arrow.
Fig. 8. Dichlorofluorescin oxidation by AS. Reaction mixtures contained 100 mM sodium phosphate, 50 μM DTPA, pH 7.4. Line 1, DCF present at 10 μM, then AS added to 20 μM to start the reaction, then Cu,Zn-SOD to 30 μg/ml at the first arrow and catalase to 260 units/ml at the second arrow. Line 2, conditions were the same as in line 1 except that AS was added to 50 μM to the reaction mixture, which had been purged with argon for 20 min, then the reaction mixture was aerated at the arrow.

DISCUSSION

The sum of Reactions 1 plus 2 is as follows.

\[ \text{NO}^+ + \text{O}_2^- + 2\text{H}^+ \rightleftharpoons \text{NO} + \text{H}_2\text{O}_2 \]

REACTION 3

In essence this is an expression of the superoxide reductase activity (5) of Cu,Zn-SOD and is in accord with Murphy and Sies (6). It should be noted that Cu,Zn-SOD was not inactivated by incubation with 0.25 mM AS for 50 min (not shown). NO⁻ / HNO can oxidize NADPH anaerobically as well as aerobically (22, 23). The anaerobic reaction presumably involves the divalent oxidation of NADPH and the concomitant reduction of HNO to NH₂OH, while the aerobic reaction could additionally be due to univalent oxidation of NADPH by ONOO⁻ formed from the rapid reaction of NO⁻ with O₂ (24, 29).

There are reasons for believing that nitric-oxide synthase can produce both NO⁻ and O₂⁻, particularly when tetrahydrobiopterin is limiting (30–32), and the observation that Cu,Zn-SOD increases the yield of NO produced per NADPH consumed could be partially explained by the superoxide reductase activity of Cu,Zn-SOD as expressed in Reaction 3.

Nitric-oxide synthase plus NADPH and l-arginine produces more O₂⁻ in the absence of tetrahydrobiopterin (32). That can partially be explained on the basis of NO⁻ production. Thus the NO⁻ plus O₂⁻ would yield ONOO⁻, which would oxidize NADPH to NAPD⁺, and that in turn would reduce O₂⁻ to O₂. Hence the stoichiometry of NADPH consumed per NO⁻ produced, by nitric-oxide synthase, could be influenced by SOD both on the basis of the oxidation of NO⁻ to NO and by the prevention of the nonenzymatic oxidation of NADPH by NO⁻ / ONOO⁻, in agreement with Reif et al. (22).

It has been suggested by Stoyanovsky et al. (33) that HNO can dimerize and then decompose to N₂ + 2H₂O. If this were the case under our conditions, then NADPH oxidation should have been appreciably due to HO⁻ and should have been noticeably inhibited by an HO⁻ scavenger such as ethanol. However ethanol, added to 2% of the reaction volume, did not at all inhibit the oxidation of NADPH by NO⁻ (not shown). Having previously noted that the oxyethyl radical does not oxidize NADPH (34) we can now exclude HO⁻ from having a role in the oxidation NADPH by NO⁻ under our conditions. It should be noted that Stoyanovsky et al. (33), using spin trapping, observed maximal HO⁻ production from HNO at pH 5, but very little at neutrality, while we worked at pH 7.4.

The reduction of the Cu(II) at the active site of Cu,Zn-SOD by NO⁻ is viewed as a reversible reaction, in accord with Murphy and Sies (6). That being said, the observation of Estevez et al. (12) might now be explained in terms of the reduction of NO to NO⁻ by the ascorbate-reduced Cu-SOD, rather than by the reduction of O₂⁻ to O₂. The NO⁻, thus produced, would lead to formation of ONOO⁻ that would account for the oxygen-dependent DCF oxidation that they observed. Another possible explanation is based on the observations of McBride et al. (16) who suggested that O₂⁻ derived from the oxidation of H₂O₂ by the Cu(II) of Cu,Zn-SOD, might then react with NO, yielding ONOO⁻ that could oxidize DHR. In the work of Estevez et al. (12) the autoxidation of ascorbyl radical is another possible source of O₂⁻ and that, in the presence of NO, would yield ONOO⁻.

The differences in reactivity between singlet and triplet NO⁻ adds complexity to the data presented herein and to that in the literature (8, 9, 18, 23, 24). Thus GSH may be oxidized by ¹NO⁻ and not by ³NO⁻ (9). Hence if the biologically generated NO⁻ is in the triplet state then GSH might not be as effective a scavenger of NO⁻ as proposed by Miranda et al. (18). The oxidations of NADPH and of DCF could have been due to NO⁻ or to ONOO⁻ derived from ¹NO⁻. NO⁻ is a small anion, akin to O₂⁻, and should readily gain access to the active site of SOD, but the possibility of SOD reacting with ONOO⁻ cannot be excluded. If the effect of SOD was mainly due to reaction with NO⁻, we can now attempt to estimate the rate constant for that interaction. The NO⁻ produced by the decomposition of AS is said to be ¹NO⁻ (18), which can relax to ⁰NO⁻ and that, in turn, reacts with ³O₂⁻ to yield ONOO⁻. We have shown that ~2 × 10⁻⁶ M SOD 50% inhibited the oxidation of NADPH, or of DCF, by AS. Suppose that SOD only reacts with ¹NO⁻ and not with ³NO⁻. Then 2 × 10⁻⁶ SOD must halve the [¹NO⁻] in competition with its conversion to ³NO⁻, plus its reactions with all targets for ¹NO⁻ attack. If there are no scavengers for ¹NO⁻ or if we disregard them, we will get a minimal estimate for the rate constant and we can write: k₁ [SOD] [¹NO⁻] = k₂ [³NO⁻], where k₁ is the rate constant for the oxidation of ¹NO⁻ by SOD, and k₂ is the rate of relaxation of ¹NO⁻ to ³NO⁻, then k₁ [SOD] = k₂. The rate constant for the relaxation of ¹NO⁻ is not known, but we can assume that it is similar to that for the relaxation of ³O₂⁻, which is of the order of 2 × 10⁶ s⁻¹ (35). Then k₁ = 10¹ⁱ M⁻¹ s⁻¹. This is unrealistically fast and might suggest that the relaxation of ¹NO⁻ is slower than that of ³O₂⁻. But even if k₁ was 2 × 10⁹ s⁻¹ the value of k₂ would be 10¹¹ M⁻¹ s⁻¹ and that is comparable with the rate constant for the reaction of ³O₂⁻ with SOD.

Now suppose that SOD reacts with ³NO⁻ and not with ¹NO⁻. In that case the SOD would be competing with the reaction of ³NO⁻ with O₂ and any other scavengers for ³NO⁻. If we again ignore reactions with other targets we can write: k₁ [SOD][³NO⁻] = k₃ [³O₂⁻][³O₂] or k₁ [SOD] = k₃ [³O₂].

[SOD] is 2 × 10⁻⁶ M and [³O₂] in aqueous solutions equilibrated with air is ~2 × 10⁻⁴ M. Two values have been reported for k₃ and these are 4 × 10⁻¹⁴ M⁻¹ s⁻¹ (29) and 7 × 10⁻¹⁴ M⁻¹ s⁻¹ (24). If we take the lower value we get k₁ = 4 × 10⁹ s⁻¹, and this is again a minimal estimate. Thus whether SOD reacts with ¹NO⁻ or ³NO⁻, or both, its rate constant must be very high to account for its observed ability to inhibit the oxidations caused by NO⁻. Whether this has relevance in the biological milieu is not yet known.

There is uncertainty in the literature concerning the pK₅₅
redox potential, and even the spin state of NO− in aqueous solution. That being the case we must explain repeatable observations as best we can and hope that, in time, the physical chemistry and theoretical treatments of this fascinating molecule will yield certain results, which in turn, will lead to refinements of those currently reasonable explanations. It should be noted that we were not necessarily dealing with free NO/NO− but rather with these species at the active site of SOD, and binding may substantially change their physicochemical character. Thus the interaction of NO with reduced SOD could give rise to a bound nitroxyl, which can be represented by the following equilibrium: Enz-Cu(I)NO → Enz-Cu(II)NO− (where Enz indicates enzyme) and which could react with O2 yielding ONOO− that could diffuse into the bulk solvent and there oxidize DCF or DHR. A similar scenario applies to the results of Sharpe and Cooper (36) who reported that aerobic ferrocyanochrome c plus NO oxidized DHR. This recalls the proposal made by Estevez et al. (12) with respect to the putative bound O2− made by the autoxidation of Cu(I)-SOD.

Note Added in Proof—The oxidation of NAD(P)H by ONOO−, with subsequent production of O2− by autoxidation of NAD(P)H, has been described by Kirsch and de Groot (37).

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