Pulse Radiolytic Investigations of Superoxide Catalyzed Disproportionation. Mechanism for Bovine Superoxide Dismutase[1a]

Dina Klug-Roth, Irwin Fridovich,[1b] and Joseph Rabani*

Contribution from the Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91000, Israel.
Received July 27, 1972

Abstract: Bovine superoxide dismutase has been subjected to pulse radiolysis in the presence of O_2, EDTA, and formate. The reaction mechanism was investigated by means of optical measurements at 650 and 300 nm, in the presence and in the absence of catalase. The results can be explained on the basis of several reactions involving the enzyme. These are: (a) $E^0 + O_2^- \rightarrow E^- + O_2$, $k = (1.2 \pm 0.2) \times 10^9\ M^{-1}\ sec^{-1}$; (b) $E^- + O_2^- (+2H^+) \rightarrow E^0 + H_2O_2$, $k = (2.2 \pm 0.4) \times 10^9\ M^{-1}\ sec^{-1}$; (c) $E^- + O_2^- \rightarrow E^{2-} + O_2$, $k = (0\text{–}3) \times 10^8\ M^{-1}\ sec^{-1}$; (d) $E^{2-} + O_2^- (+2H^+) \rightarrow E^- + H_2O_2$, $k = (1.2 \pm 0.2) \times 10^9\ M^{-1}\ sec^{-1}$; (e) $E^0 + H_2O_2 \rightarrow E^{2-} + 2H^+ + O_2$; (f) $E^- + O_2 \rightarrow E^0 + O_2^-$, $k = 0.44 \pm 0.12\ M^{-1}\ sec^{-1}$. E^0 refers to the native enzyme in which both copper atoms are oxidized while E^- and E^{2-} refer to the singly and doubly reduced enzyme, respectively. Reactions a and b account for the activity of the enzyme in the absence of H_2O_2, whereas reactions a, b, and d account for the activity of the modified form of the enzyme which was generated by reaction with H_2O_2, as in reaction e. Reaction f describes the slow reoxidation of reduced enzyme which was observed in the presence of O_2.

Superoxide radical is known[2] to be produced by a number of biologically significant oxidations and is probably generated, to some degree, in all oxygen metabolizing cells. Superoxide dismutase (SOD), which catalyzes the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$,[3] appears to be an important component of the defenses which have been evolved to deal with the cytotoxicity of this radical.[4] This enzyme is a metalloprotein which has been found to contain Cu^{2+} and Zn^{2+} in eucaryotes and manganese in procaryotes.[2] Pulse radiolysis has already been used in studies of reactivity of the eucaryotic SOD[5–7] and promises to be a powerful tool in the elucidation of its mechanism of action.

Irradiation of oxygenated aqueous solutions, in the presence of formate, results in the following reactions.[8]

$$H_2O \rightsquigarrow e_{aq}^-, H, OH, H_2, H_2O_2, H_3O^+, OH^- \quad (1)$$

$$e_{aq}^- + O_2 \longrightarrow O_2^- \qquad k = 2 \times 10^{10}\ M^{-1}\ sec^{-1}\ [9] \quad (2)$$

$$H + O_2 \longrightarrow HO_2 \qquad k = 2 \times 10^{10}\ M^{-1}\ sec^{-1}\ [10] \quad (3)$$

$$OH + HCO_2^- \longrightarrow H_2O + CO_2^- \qquad k = 2 \times 10^9\ M^{-1}\ sec^{-1}\ [11] \quad (4)$$

$$H + HCO_2^- \longrightarrow H_2 + CO_2^- \qquad k = 5 \times 10^8\ M^{-1}\ sec^{-1}\ [12] \quad (5)$$

$$CO_2^- + O_2 \longrightarrow CO_2 + O_2^- \quad (6)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \qquad K_{eq} = 1.6 \times 10^{-5}\ M\ [8,13] \quad (7)$$

It is thus possible to use pulse radiolysis for the production of O_2^- and initial concentrations of $10^{-5}\ M$ can be generated with brief pulses. In the absence of catalysts, superoxide radicals decay by a spontaneous dismutation reaction whose rate is dependent upon pH.[8,13] At pH $\simeq$7.5 the predominant uncatalyzed decay reaction is $HO_2 + O_2^- (+H^+) \rightarrow O_2 + H_2O_2$. This is the case, despite the relatively small proportion of HO_2 at this pH ($[HO_2]/[O_2^-] \simeq 10^3$), because of the rapidity of this reaction ($k = 8.5 \times 10^7\ M^{-1}\ sec^{-1}$) as compared to the dismutation among O_2^- radicals ($k(O_2^- + O_2^-) \simeq 10^2\ M^{-1}\ sec^{-1}$). It is important to note that small concentrations of impurities can introduce an apparent first-order component to the decay of O_2^-.[13]

SOD catalyzes the dismutation of O_2^- in a reaction which is first order with respect to enzyme and to O_2^- and whose rate constant at pH 7.5 is approximately $1.5 \times 10^9\ M^{-1}\ sec^{-1}$.[5–7] It has been demonstrated that the products of this enzymatic reaction are O_2 and H_2O_2.[3,14] It appeared possible to probe the mechanism of this reaction by observing the effects of pulse radiolysis upon the absorption of this enzyme at 650 nm which is due to its content of Cu^{2+}. We now report the results of such investigations.

Experimental Section

Pulse Radiolysis. The apparatus, optical system, and cell-filling technique have been described.[8] The light source was a 150-W water-cooled xenon lamp. Scattered light was less than 1% at 650 nm and was ignored. When observations were made at 650 nm, all wavelengths below 600 nm were eliminated by filters placed between the lamp and the cell to minimize photolysis and between the cell and the monochromator to absorb Cherenkov light. Time resolu-

(1) (a) This work was supported by the Authority for Research and Development of the Hebrew University of Jerusalem and by Grant GM-10287 from the American National Institutes of Health, Bethesda, Maryland. (b) Department of Biochemistry, Duke University Medical Center, Durham, N. C. 27710.
(2) I. Fridovich, *Accounts Chem. Res.*, 5, 321 (1972).
(3) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 244, 6049 (1969).
(4) J. M. McCord, B. B. Keele, Jr., and I. Fridovich, *Proc. Nat. Acad. Sci. U. S.*, 68, 1024 (1971).
(5) G. Rotilio, R. C. Bray, and E. M. Fielden, *Biochim. Biophys. Acta*, 268, 605 (1972).
(6) D. Klug, J. Rabani, and I. Fridovich, *J. Biol. Chem.*, 247, 4839 (1972).
(7) J. Rabani, D. Klug, and I. Fridovich in "Molecular Basis of Radiation Biology," G. Stein, Ed., Weizmann Press, Rehovot, Israel, 1972, p 1095.
(8) D. Behar, G. Czapski, L. M. Dorfman, J. Rabani, and H. A. Schwarz, *J. Phys. Chem.*, 74, 3209 (1970).
(9) (a) J. P. Keene, *Radiat. Res.*, 22, 1 (1964); (b) S. Gordon, E. J. Hart, M. S. Matheson, J. Rabani, and J. K. Thomas, *J. Amer. Chem. Soc.*, 85, 1375 (1963).
(10) (a) J. P. Sweet and J. K. Thomas, *J. Phys. Chem.*, 68, 1363 (1964); (b) H. Fricke and J. K. Thomas, *Radiat. Res., Suppl.*, 4, 35 (1964).
(11) M. S. Matheson, W. A. Mulac, J. L. Weeks, and J. Rabani, *J. Phys. Chem.*, 70, 2092 (1966).
(12) (a) J. Rabani, *J. Phys. Chem.*, 66, 361 (1962); (b) J. Rabani and D. Meyerstein, *ibid.*, 72, 1599 (1968).
(13) J. Rabani and S. O. Nielsen, *J. Phys. Chem.*, 73, 3736 (1969).
(14) I. Fridovich, *J. Biol. Chem.*, 245, 4053 (1970).

tion at 650 nm was 4 μsec which includes 1.5 μsec of pulse duration. The enzyme absorbs below 300 nm and the amount of scattered light depended on enzyme concentrations, light path, and optical alignment. The amount of scattered light was estimated by setting the monochromator at 180 nm, at which wavelength no signal should have been observed, in the complete absence of scatter. Tektronix 556 dual beam and Tektronix 549 memory scopes were used in parallel. One beam was used to monitor the pulse intensity by measuring the inductance current generated by passage of the electron beam through a coil. The other two beams were used for simultaneous measurements at two different sweep rates. All containers were cleaned in boiling distilled water. All measurements were made at 23°.

Materials. Water was redistilled five times. The first of these distillations was from alkaline permanganate and the second was from acid bichromate. The last two distillations were performed through heated columns to prevent ion migration. pH was measured both before and after irradiation and was found to be 7.5. SOD was prepared from bovine erythrocytes and its concentration was determined on the basis of its absorbancy at 260 nm[3] ($\epsilon = 1.13 \times 10^4\ M^{-1}\ cm^{-1}$). Sodium formate and disodium ethylenediamine tetraacetate (EDTA) were reagent grade and used without further purification. Additional experimental details have been described previously.[6]

Results and Discussion

All the experiments were carried out in 0.16 M sodium formate and 10^{-4} M EDTA in aerated or oxygenated solutions. Unless otherwise stated, pulse irradiations were carried out using previously unirradiated solutions of the enzyme.

Reduction of SOD by O_2^-. When SOD was exposed to pulse irradiation in the presence of 9.7×10^{-8} M catalase, its absorbance at 650 nm ($\epsilon = 250\ M^{-1}\ cm^{-1}$) was partially bleached (27–30%) by the first pulse. The second pulse caused slight additional bleaching (0–2%) and additional pulses given to the same solutions at a rate of at least one pulse per minute were with little or no further effect. Typical results are presented in Figure 1. Similar observations have been made by Rotilio, Bray, and Fielden.[5] The half-life of this bleaching was 10–20 μsec and 29% of the absorbance at 650 nm (on the average) was the limit of bleaching. Changing the enzyme concentration in the range $(0.3 \rightarrow 1.2) \times 10^{-5}$ M did not modify these results. Decay of the O_2^- was not accompanied by reappearance of the absorbance at 650 nm.

Suppose that SOD can be reduced and then reoxidized by O_2^- as follows.

$$E^0 + O_2^- \longrightarrow E^- + O_2 \qquad (8)$$

$$E^- + O_2^- \xrightarrow{2H^+} E^0 + H_2O_2 \qquad (9)$$

In this case a pulse which generates a sufficient amount of O_2^- will cause the enzyme to partition itself between the E^0 and E^- forms such that $k_8[E^0] = k_9[E^-]$. The limiting fraction of bleached absorbance at 650 nm will be given by

$$\Delta D_T/D_0 = (\epsilon_r^- - 1)/(1 - k_9/k_8) \qquad (10)$$

where ΔD_T is the sum of absorbance changes at 650 nm caused by rapid successive pulses, D_0 the initial absorbance at 650 nm, and ϵ_r^- the ratio of the extinction coefficients of E^- over E^0, that is, $\epsilon_{E^-}^{650}/\epsilon_{E^0}^{650}$. The need in some experiments for a second electron pulse in order to achieve the limiting value for bleaching is due to the fact that at relatively high [SOD] the amount of O_2^- generated by one pulse is not sufficient. In order to reach the limiting bleaching the amount of O_2^- must be somewhat higher than the amount of SOD.

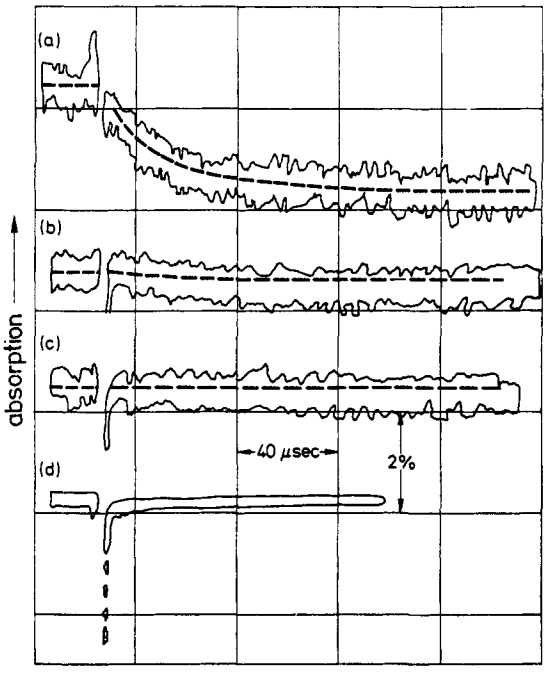

Figure 1. Oscilloscope traces at 650 nm, 9.7×10^{-6} M enzyme + 9.7×10^{-8} M catalase in 0.16 M sodium formate and 10^{-4} M EDTA, O_2 saturated; 1.5-μsec pulse of 7.3×10^{25} eV $l.^{-1}$ sec^{-1}; light path 12.3 cm. Dotted lines represent computed values using $k_8 = 1.4 \times 10^9\ M^{-1}\ sec^{-1}$, $k_9 = 2.5 \times 10^9\ M^{-1}\ sec^{-1}$, $\epsilon_{E^-}^{650} = 13\ M^{-1}\ cm^{-1}$: (a) first pulse; (b) second pulse given about 1 min after the first pulse; (c) third pulse given about 1 min after the second pulse; (d) Cherenkov signal obtained when the xenon light source was closed.

The dotted lines in Figure 1 were computed using Schmidt's program.[15] k_8 was measured from the rate of decay of O_2^- followed at 280 nm when SOD was present in great excess over O_2^- and was found to be $(1 - 1.4) \times 10^9\ M^{-1}\ sec^{-1}$. Five independent best fits of the data to assumed values of k_8 and k_9 gave $k_8 = (1.2 \pm 0.2) \times 10^9\ M^{-1}\ sec^{-1}$, $k_9 = (2.2 \pm 0.5) \times 10^9\ M^{-1}\ sec^{-1}$, and $\epsilon_{E^-}^{650} = 50 \pm 50\ M^{-1}\ cm^{-1}$. The computed extinction coefficient for E^- was very sensitive to small changes in both k_9/k_8 and $\Delta D_T/D_0$. Results in the absence of catalase, as well as at lower enzyme concentrations (up to a factor of 3 has been checked), are the same within experimental error. In all of these experiments doses ranging from 1.1×10^{20} to 1.35×10^{20} eV $l.^{-1}$ per pulse were employed. It is noted in Figure 1 that the computed curves for the second and third pulses do not fit the data as well as was the case for the first pulse. This was due to a slow oxidation of E^- by O_2 and will be further explored in the following section. Because of this effect, k_9 was computed on the basis of fits to the absorbance change during the first pulse only, and ΔD_T was also obtained from the bleaching caused by the first pulse and was corrected by adding 5 to 10%. Essentially the same results have been obtained in oxygenated as well as air-saturated solutions.

Oxidation of Reduced SOD by O_2. The data in Figure 1 were obtained by applying multiple pulses within a short time. Different results were obtained when the solutions were allowed to stand for a relatively long period between pulses. Thus, as illustrated in Figure 2, there was a regeneration of the phenomenon

(15) K. H. Schmidt, ANL Report 7199, Argonne National Laboratory, Argonne, Ill., 1966.

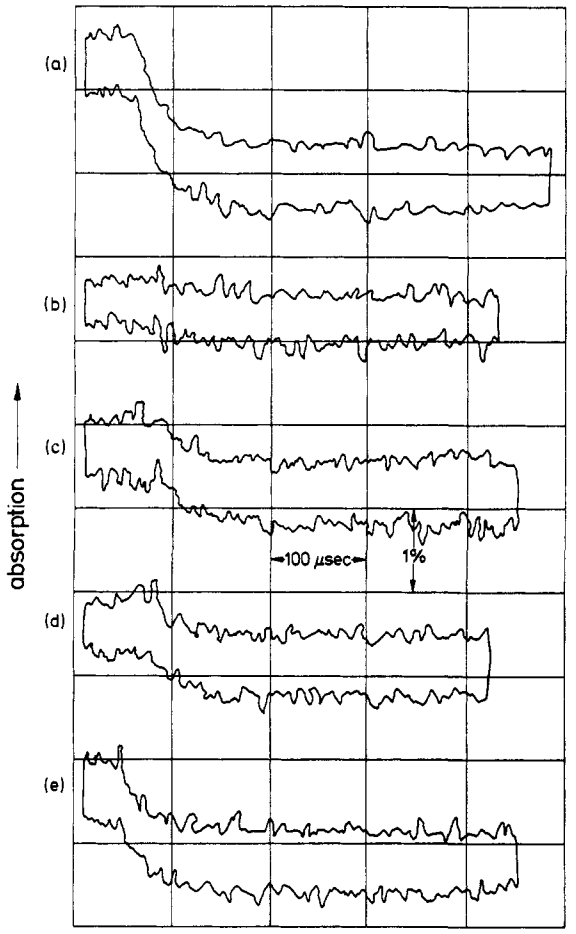

Figure 2. Oscilloscope traces at 650 nm; repetitive pulses in aerated and oxygenated solutions: 6.6×10^{-6} M superoxide dismutase + 9.7×10^{-8} M catalase in 0.16 M sodium formate and 10^{-4} M EDTA solution; 1.5-μsec pulse (7.6×10^{25} eV l.$^{-1}$ sec^{-1}); light path 12.3 cm. (a) O_2 saturated, first pulse (identical results obtained with aerated solutions). (b) Aerated, the solution was pulse irradiated and later allowed to stand 27 min before taking this trace. (c) Aerated, time interval was 66 min. (d) Oxygenated, time interval was 14 min. (e) Oxygenated, time interval was 32 min.

of decay of absorbance after an electron pulse. The magnitude of the regenerated ΔD_t (the absorbance change observed after an interval t in pulse irradiation) depends on the time which the solution was allowed to stand and on the oxygen concentration. We attribute this to a slow reversal of the bleaching at 650 nm. This return of color was greater in oxygen-equilibrated solutions than in air-equilibrated solutions. These results suggest that reduced SOD can be reoxidized by O_2 as follows:

$$E^- + O_2 \longrightarrow E^0 + O_2^- \qquad (-8)$$

In this case the O_2^- generated by reaction −8 would participate in reactions 8 and 9, and one could write

$$-d[E^-]/dt = k_{-8}[E^-][O_2] + (k_9[E^-] - k_8[E^0])[O_2^-] \qquad (11)$$

If we assume a steady state for $[O_2^-]$ during the slow reoxidation of the E^- and insert $[E^0] = [E]_{total} - [E^-]$, then by integration and rearrangement we obtain

$$k_8[E]_{total}(1/[E^-]_t - 1/[E^-]_0) + (k_9 - k_8) \ln [E^-]_0/[E^-]_t = 2k_{-8}k_9[O_2]t \qquad (12)$$

where $[E]_{total}$ is the total enzyme concentration, $[E^-]_t$ the concentration of the reduced enzyme at time t, and $[E^-]_0$ the concentration of the reduced enzyme at time zero. Note that zero time is the time when E^- and E^0 are at their limiting concentrations as determined by $k_8[E^0] = k_9[E^-]$. Except when nearly all of the enzyme is in the form of E^0, one pulse generates $[O_2^-]$ which is sufficient to bring the system back to the limiting concentrations of E^- and E^0. The absorbance change in a given pulse is attributed to the reoxidation of E^- by O_2 (reaction −8). Hence

$$\Delta D_t = ([E^-]_t - [E^-]_0)(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650})l \qquad (13)$$

If $[E^-]_t$ from eq 13 is substituted into eq 12, and if $[E^-]_0$ is replaced by $-\Delta D_{fp}/(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650})l$ we get eq 14,

$$(k_8[E]_{total}l/2k_9)(1/\Delta D_{fp} - 1/(\Delta D_{fp} - \Delta D_t)) + ((k_9 - k_8)/2k_9(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650})) \ln (\Delta D_{fp}/(\Delta D_{fp} - \Delta D_t)) = k_{-8}[O_2]t/(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650}) \qquad (14)$$

where ΔD_{fp} is the total absorbance change at 650 caused by two electron pulses given within 1 min to a solution which initially contains only E^0 and l is the light path.

Equation 14 allows the calculation of k_{-8}. Values calculated in this way are given in Table I. The

Table I. Evaluation of k_{-8} at 23°

Time, min	$10^6[E]_{total}$, M	$10^3 \cdot \Delta D_t$	$10^3 \cdot \Delta D_{fp}$	$10^3[O_2]$, M	k_{-8}, M^{-1} sec^{-1}
2	9.60	0.9	9.1	1.35	0.63
2	6.65	0.4	6.4	1.35	0.38
4	6.65	0.9	6.4	1.35	0.46
7	6.65	1.2	6.4	1.35	0.37
7	6.65	1.5	6.4	1.35	0.48
8	6.65	1.6	6.4	1.35	0.46
10.5	9.70	1.8	9.1	1.35	0.27
11	6.65	2.1	6.4	1.35	0.48
14	6.65	2.3	6.4	1.35	0.43
14	6.65	0.4	6.4	0.27	0.27
15	6.65	0.4	6.4	0.27	0.25
19	7.30	0.7	6.9	0.27	0.32
19	6.65	3.2	6.4	1.35	0.55
19.5	9.70	4.2	9.1	1.35	0.48
20	6.65	3.0	6.4	1.35	0.47
24	6.65	0.9	6.4	0.27	0.38
25	6.65	3.6	6.4	1.35	0.53
28	9.70	5.1	9.1	1.35	0.48
32	7.30	1.7	6.9	0.27	0.57
32	6.65	4.3	6.4	1.35	0.65
38	7.30	1.2	6.9	0.27	0.31
66	6.65	2.5	6.4	0.27	0.53

results give $k_{-8} = 0.44 \pm 0.12$ M^{-1} sec^{-1} (mean deviation). Using the experiments in the oxygenated solutions alone, an average $k_{-8} = 0.47 \pm 0.1$ M^{-1} sec^{-1} results. In aerated solutions $k_{-8} = 0.37 \pm 0.15$ M^{-1} sec^{-1}. The calculations were based on $(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650}) = 200$ M^{-1} cm^{-1}, $k_8 = 1.2 \times 10^9$, and $k_9 = 2.2 \times 10^9$ M^{-1} sec^{-1}. The second term on the left-hand side of eq 14 contributed only about 25% to the value of k_{-8}. Therefore, the results are not expected to show great sensitivity to the ratio k_9/k_8 chosen within the reasonable error limits. *E.g.*, if $k_9/k_8 = 1$ is assumed, $k_{-8} = 0.64$ M^{-1} sec^{-1} results, as compared with a value of 0.44 M^{-1} sec^{-1} calculated for $k_9/k_8 = 1.3$. The values calculated for k_{-8} are nearly inversely proportional to the values chosen for $(\epsilon_{E^0}{}^{650} - \epsilon_{E^-}{}^{650})$. Thus, assuming

that E^- does not absorb light at 650 nm, we find $k_{-8} = 0.53\ M^{-1}\ sec^{-1}$. Assuming $\epsilon_{E^-}{}^{650} = 100\ M^{-1}\ cm^{-1}$ which is the upper limit (see previous paragraph), we obtain $k_{-8} = 0.36\ M^{-1}\ sec^{-1}$. The error limits for k_{-8} do not include systematic errors due to deviations in $[O_2]$ and in temperature.

Bleaching of SOD at 650 nm by H_2O_2. All the work described in this section has been carried out in oxygenated solutions using 1.5-μsec electron pulses (producing about $10^{-5}\ M\ O_2^-$ per pulse). Equilibration with O_2 was carried out after each set of 20 pulses. There was no effect of $[O_2]$ on the results described here. In some experiments air was used instead of oxygen. The dismutase concentration was varied between 0.4×10^{-5} and $1.1 \times 10^{-5}\ M$.

The first two pulses gave results similar to those obtained when catalase was absent (see Figure 1). However, the effect of additional pulses was different. Under these circumstances a 1.5 μsec pulse, which followed upon a series of (10–100) similar pulses, actually caused an increase in absorbance at 650 nm. The magnitude of this change was positively dependent upon the number of preceding pulses and upon the time which elapsed between the last of the preceding pulses and the test pulse. A limiting value was obtained by preirradiating with 30 pulses and incubating 1 min prior to the test pulse. This limiting value was proportional to the concentration of enzyme: $\Delta D = 87[E]_{total}l$. This effect is illustrated in Figure 3. It is apparent that this increase in absorbance at 650 nm occurred on a time scale comparable to the decreases in absorbance which had previously been seen in catalase-containing solutions (Figures 1 and 2). The enzyme remained active as a superoxide dismutase toward pulses of O_2^-, even after H_2O_2 had accumulated to $5 \times 10^{-4}\ M$ with very little, if any, change in activity.

H_2O_2 has been reported[16] to decrease the intensity of the esr signal of SOD when incubated with the enzyme in the absence of oxygen. Subsequent aeration caused a slow return of the original signal. This suggests that the Cu^{2+} in SOD can be reduced by H_2O_2. We propose that H_2O_2 can reduce SOD beyond the level of E^- as follows

$$E^0 + H_2O_2 \longrightarrow E^{2-} + 2H^+ + O_2 \qquad (15)$$

and that E^{2-} can be reoxidized by O_2^- as follows.

$$E^{2-} + O_2^- \xrightarrow{2H^+} E^- + H_2O_2 \qquad (16)$$

It appears possible that E^{2-} can also be generated by a reaction of E^- with O_2^- as follows

$$E^- + O_2^- \longrightarrow E^{2-} + O_2 \qquad (17)$$

but this reaction must be much slower than reaction 9 in which E^- is reoxidized to E^0 by its encounter with O_2^-. The proposal then is that H_2O_2, when present in sufficient concentration, converts SOD to the E^{2-} from within 1 min after the O_2^- has decayed away and that E^{2-} has less absorbance at 650 nm than the mixture of E^0, E^-, and E^{2-} which results from the interactions of the enzyme with O_2^-. The test pulse of O_2^- then largely eliminates E^{2-} by reaction 16 and thus causes the increase in absorbance shown in Figure 3. Note that the experiments reported so far can also be interpreted in terms of E^0 and E^- alone. The reasons for invoking E^{2-} and the rates of reactions 16 and 17 will be given in the next paragraphs.

(16) G. Rotilio, L. Calabrese, F. Bossa, D. Barra, A. Finazzi Agro, and B. Mondovi, *Biochemistry*, 11, 2182 (1972).

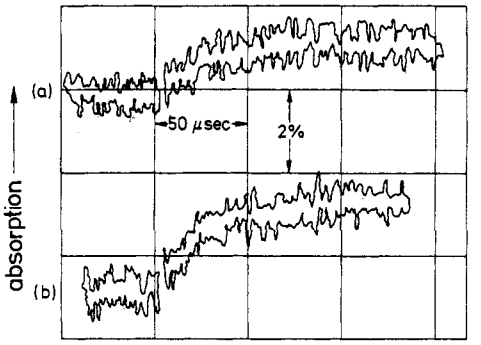

Figure 3. Oscilloscope traces at 650 nm (no catalase): $8.5 \times 10^{-6}\ M$ superoxide dismutase in 0.16 M sodium formate and $10^{-4}\ M$ EDTA; aerated; light path 12.3 cm; 1.5-μsec pulse producing $1.0 \times 10^{-5}\ M\ O_2^-$ ($G = 6$) per pulse. Equilibration with air was made after every 20th pulse. (a) Fifth pulse taken 13 min after the previous pulse. (b) 95th pulse taken 13 min after the previous pulse.

Bleaching of SOD at 300 nm by H_2O_2. When oxygenated solutions of SOD in 0.16 M sodium formate and $10^{-4}\ M$ EDTA were subjected to 0.05-μsec pulses (5×10^{18} eV $l.^{-1} \pm 20\%$), the absorbance at 300 nm was increased due to the generation of O_2^-. In solutions which had not been previously irradiated, this absorbance rapidly decayed to a level somewhat lower than the initial absorbance due to the enzyme. If however, the solution had been subjected to a series of high intensity (1.3×10^{20} eV $l.^{-1}$) pulses to accumulate H_2O_2 in the solution (catalase absent) and then, after a delay of at least 1 min, was tested with a low intensity pulse, the absorbance at 300 nm increased and this increase did not decay away within the time of our measurements. This difference between solutions which had not been previously irradiated and those which were previously irradiated is shown in Figures 4a and 4b. On the other hand, rapid pulsing (10 pulses/sec) of a solution which had been preirradiated and then incubated for 1 min did restore the phenomenon of decay. This is shown in Figure 4c.

If we ascribe the increase at 300 nm, which accompanied pulsing, to O_2^-, then the decay shown in Figure 4a was due mainly to reaction 8. The negative plateau seen in Figure 4a can be explained on the basis of the conversion of some E^0 to E^- and on the assumption that $\epsilon_{E^0}{}^{300} > \epsilon_{E^-}{}^{300}$. When the enzyme had been incubated with H_2O_2, generated by preirradiation, the increase in absorbance at 300 nm did not decay rapidly (Figure 4b). This is explained by the assumption that the decrease in absorbance at 300 nm, due to the decay of O_2^-, was compensated by an equal and equally rapid increase in absorbance due to a net reoxidation of E^{2-} by O_2^- as in reaction 16. Rapid pulsing (10 pulses/sec) of a solution in which H_2O_2 had accumulated would restore the steady-state balance of E^0, E^-, and E^{2-} and would then allow the decay of O_2^- to be seen at 300 nm because the excess of E^{2-} had been eliminated by the preceding rapid pulses of O_2^-. This is shown in Figure 4c and was made possible by the relative slowness of reaction 15.

One might argue that H_2O_2 reduces E^0 to E^- rather than to E^{2-}. In this case the result shown in Figure 4b

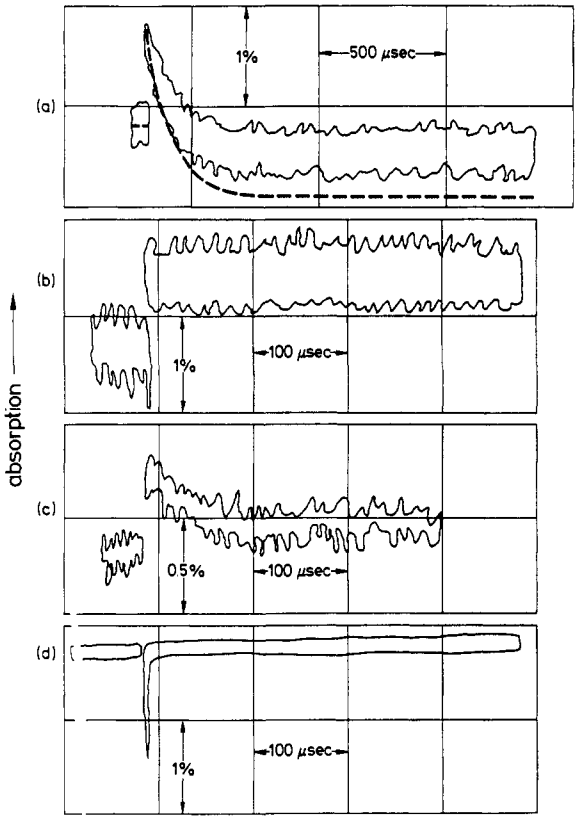

Figure 4. Oscilloscope traces at 300 nm, 0.16 sodium formate and 10^{-4} M EDTA. (a) First 0.1-μsec pulse (9×10^{25} eV $l.^{-1}$ sec^{-1}). An oxygenated solution containing 7.1×10^{-6} M superoxide dismutase. The dotted line is computed on the basis of the two step mechanism, $k_8 = 1.4 \times 10^9$ M^{-1} sec^{-1}, $k_9 = 2.8 \times 10^9$ M^{-1} sec^{-1}, $\epsilon_{E^0}^{300} = 2220^2$ M^{-1} cm^{-1}, $\epsilon_{O_2^-}^{300} = 350^7$ M^{-1} cm^{-1}, $\epsilon_{E^-}^{300} = \epsilon_{E^0}^{300} - \epsilon_{O_2^-}^{300} = 1870$ M^{-1} cm^{-1}. The final plateau does not depend on the values taken for k_8 and k_9, within their error limits. The light path was 12.3 cm, scattered light 5%. (b) Same solution as in (a) was pulse irradiated using 50 pulses, each of 1.5-μsec duration 1.30×10^{20} eV $l.^{-1}$ per pulse. Ten minutes later this trace has been taken with a 0.1-μsec pulse, 9×10^{18} eV $l.^{-1}$. (c) Eighty-first pulse (about 0.05 μsec, 5×10^{16} eV $l.^{-1}$ per pulse). The 81 pulses were taken at a rate of 10 pulses per second. The enzyme solution has been preirradiated using 50 pulses of 1.5 μsec (1.3×10^{20} eV $l.^{-1}$ per pulse). Three minutes separate the last of the 50 preirradiation pulses and the first of the series of 81 pulses. $[E]_{total} = 2.5 \times 10^{-5}$ M. The light path was 4 cm, scattered light 10%. (d) No light source being used.

could be explained on the basis that

$$\epsilon_{E^0}^{300} - \epsilon_{E^-}^{300} \simeq \epsilon_{O_2^-}^{300} \qquad (18)$$

However, the dotted line in Figure 4a was computed on the basis of this assumption and its failure to agree with the results is obvious. Such disagreements were found to be typical. SOD concentrations ranging from 7 to 25 μM have been tested. It was always found that while the computations gave $D_0/D_{plateau} \simeq 1$, experiments gave values of about 4. For this reason the product of the reduction of E^0 by H_2O_2 is taken to be E^{2-}. It is, of course, possible that E^- is a transient intermediate in the reduction of E^0 to E^{2-} by H_2O_2.

Discussion of the Four-Step Mechanism. The method used for the computation of k_8 and k_9, based on Schmidt's program,[15] can now be used for the evaluation of k_9, k_{16}, and k_{17}. This method depends upon comparison of computed and experimental absorbances using known or assumed rate constants and extinction coefficients. Of the extinction coefficients $\epsilon_{E^0}^{650} = 250$ M^{-1} cm^{-1} has been reported previously.[3] $\epsilon_{E^{2-}}^{650} \simeq 80$ M^{-1} cm^{-1} was calculated on the basis of our postulated reactions 8, 9, 16, and 17. Use was made of the fact that the same steady-state concentrations of E^0, E^-, and E^{2-}, and consequently the same absorbances, are produced during the O_2^- decay in solutions containing initially E^0 only and in solutions initially containing only E^{2-}. The reaction rate constants $k_8 = (1.2 \pm 0.2) \times 10^9$ M^{-1} sec^{-1} and $k_{16} = (1.2 \pm 0.2) \times 10^9$ M^{-1} sec^{-1} were measured from the decay of the O_2^- absorbance at 280 nm when excess of E^0 and of E^{2-} were respectively present. Thus, of the extinction coefficients and rate constants involved, only k_9, k_{17}, and $\epsilon_{E^-}^{650}$ had to be assumed. About 50 computations were carried out, in which these parameters varied systematically. Agreement with the experimental data was obtained when k_{17} ranged from (0 to 3) $\times 10^8$ M^{-1} sec^{-1}. Values of k_9 which were in agreement ranged between (1 to 3) $\times 10^9$ M^{-1} sec^{-1}.

These results have been cross checked at 300 nm. An apparent reaction rate constant "k," which is defined as $(1/[E]_{total})$ d ln $[O_2]/dt$ was evaluated on the basis of traces similar to that shown in Figure 4c and found to be "k" $= (1.4 \pm 0.3) \times 10^9$ M^{-1} sec^{-1}. If a steady state can be assumed for E^0, E^-, and E^{2-} under the conditions of Figure 4c, then

$$\text{"}k\text{"} = 2(k_9 + k_{17})/(1 + k_9/k_8 + k_{17}/k_{16}) \qquad (19)$$

Inserting $k_8 = 1.2 \times 10^9$, $k_{16} = 1.2 \times 10^9$, and $k_{17} =$ (0 to 0.3) $\times 10^9$ M^{-1} sec^{-1} in eq 19 gives $k_9 =$ (1.4 to 1.7) $\times 10^9$ M^{-1} sec^{-1}. This value is in agreement with the values of k_9 ranging between 1×10^9 and 3×10^9 M^{-1} sec^{-1} obtained from the measurements at 650 nm. The value $k_9 =$ (1.4 to 1.7) $\times 10^9$ M^{-1} sec^{-1} is also in fair agreement with $k_9 = 2.2 \times 10^9$ M^{-1} sec^{-1} which could be directly estimated from traces such as those in Figure 1 on the basis of neglect of reactions 16 and 17. The fact that k_{17} is relatively small justified this neglect. If $k_{17} = 0$ then the mechanism involves reactions 8 and 9 only, as long as the SOD was added to the reaction mixture in the form of E^0 and no H_2O_2 was allowed to accumulate. In fact, k_{17} may be as high as 3×10^8 M^{-1} sec^{-1}. In such case, the value of $k_9 = 2.2 \times 10^9$ M^{-1} sec^{-1} will have to be slightly decreased (by 10–20%). We conclude that the mechanism, based upon reactions 8 and 9 only, is at least a good approximation for the SOD reaction in solutions which contain catalase or in which the accumulation of H_2O_2 is somehow prevented. When H_2O_2 does accumulate reaction 16 becomes important in addition to reactions 8 and 9.

Acknowledgment. The authors are indebted to Mr. Y. Ogdan for careful Liniac operations, and to Professors G. Czapski, A. Henglein, M. S. Matheson, and G. Stein for helpful comments and discussion.