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The Catalytic Mechanism of the Manganese-Containing Superoxide Dismutase of *Escherichia coli* Studied by Pulse Radiolysis[1a]

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Abstract: The dismutation of O_2^-, catalyzed by *Escherichia coli* Mn dismutase, has been investgated. O_2^- was generated in formate aqueous solutions by pulse radiolysis. When the initial concentration of O_2^-, $[O_2^-]_0$, is less than 10 times the total concentration of the dismutase, $[E]_0$, a reaction first order in both $[O_2^-]$ and $[E]_0$ is observed, the apparent reaction rate constant of which is $1.5 \pm 0.15 \times 10^9\ M^{-1}\ sec^{-1}$. When $[O_2^-]_0/[E]_0 > 15$, a biphasic process is observed. Under these conditions only about 15 O_2^- radical ions per each dismutase molecule react with a relatively fast rate. Excess O_2^- is removed by a less efficient reaction, also first order in $[E]_0$ and nearly first order in $[O_2^-]$, which has an apparent rate constant $1.6 \pm 0.25 \times 10^8\ M^{-1}\ sec^{-1}$. The results are interpreted in terms of four oxidation and reduction reactions, such as: (i) $E + O_2^- \rightarrow E^- + O_2$, $k = 1.3 \pm 0.15 \times 10^9\ M^{-1}\ sec^{-1}$; or (i)′ $E + O_2^- + 2H^+ \rightarrow E^+ + H_2O_2$; (ii) $E^- + O_2^- + 2H^+ \rightarrow E + H_2O_2$, $k = 1.6 \pm 0.6 \times 10^9\ M^{-1}\ sec^{-1}$; or (ii)′ $E^+ + O_2^- \rightarrow E + O_2$; (iii) $E^- + O_2^- \rightarrow E^{2-} + O_2$, $k \simeq 2 \times 10^8\ M^{-1}\ sec^{-1}$; or (iii)′ $E + O_2^- \rightarrow E^- + O_2$; (iv) $E^{2-} + O_2^- + 2H^+ \rightarrow E^- + H_2O_2$, $k \simeq 1 \times 10^7\ M^{-1}\ sec^{-1}$; or (iv)′ $E^- + O_2^- + 2H^+ \rightarrow E + H_2O_2$. E^+, E, E^-, and E^{2-} may represent forms of enzymes in which Mn^{IV}, Mn^{III}, Mn^{II}, and Mn^{I} are respectively present. Alternative mechanisms involving additional oxidation states of the Mn are discussed. After the end of the catalytic process the high enzyme activity is fully regenerated within less than 30 sec.

Enzymatic catalysis of superoxide dismutation has been evident since the work of McCord and Fridovich.[2] The rate of catalysis and its mechanism have been extensively investigated for bovine superoxide dismutase.[3-6] It has been shown that alternate reduction and oxidation of the copper atoms is involved.[6]

Recently, competition experiments have shown a high catalytic efficiency of *Escherichia coli* mangano superoxide dismutase.[7] In this manuscript, we report a direct observation of the catalysis, followed with the aid of the pulse radiolysis technique.

When an oxygenated aqueous solution containing formate ions is irradiated the following reactions take place.

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

$$e_{aq}^- + O_2 \longrightarrow O_2^- \quad k_2 = 2 \times 10^{10}\ M^{-1}\ sec^{-1}\ [8] \quad (2)$$

$$H + O_2 \longrightarrow HO_2 \quad k_3 = 2 \times 10^{10}\ M^{-1}\ sec^{-1}\ [9] \quad (3)$$

$$H + HCO_2^- \longrightarrow H_2 + CO_2^- \quad k_4 = 5 \times 10^8\ M^{-1}\ sec^{-1}\ [10] \quad (4)$$

$$OH + HCO_2^- \longrightarrow H_2O + CO_2^- \quad k_5 = 3 \times 10^9\ M^{-1}\ sec^{-1}\ [11] \quad (5)$$

$$CO_2^- + O_2 \longrightarrow CO_2 + O_2^-\ [12] \quad (6)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \quad K = 1.6 \times 10^{-5}\ M\ [13,14] \quad (7)$$

As a result, at sufficiently high [formate], O_2^- radical ions (in equilibrium with HO_2) are produced as the only radical species within less than 1 μsec. O_2^- radical ions decay away to form O_2 and H_2O_2. Under our conditions, reaction 8 competed efficiently with both (9) and (10). In the following we report the enhanced decay of the O_2^- radical ions, as followed in the uv, upon the addition of the *E. coli* Mn dismutase.

$$HO_2 + O_2^- \xrightarrow{H^+} H_2O_2 + O_2 \quad k_8 = 8.5 \times 10^7\ M^{-1}\ sec^{-1}\ [13,14] \quad (8)$$

$$HO_2 + HO_2 \longrightarrow H_2O_2 + O_2 \quad k_9 = 6.7 \times 10^5\ M^{-1}\ sec^{-1}\ [13,14] \quad (9)$$

$$O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + O_2 \quad k_{10} \cong 10^2\ M^{-1}\ sec^{-1}\ [14] \quad (10)$$

Experimental Section

The pulse radiolysis apparatus and the optical detection system have been described previously.[14] The solutions contained 10^{-4} *M* ethylenediaminetetraacetate (EDTA) and 10^{-2} *M* formate unless otherwise stated. Blank experiments were always carried out before the injection of the enzyme from a stock solution. In the blank solutions, O_2^- decayed away by two parallel reactions. One of these was a second-order process and the other an apparent first-order process. Similar observations were reported and discussed previously.[13] These results indicate a fairly low level of impurities in our solutions. Phosphate buffer was used to adjust the pH. *E. coli* dismutase has been isolated and purified as before.[15] Other materials were of high purity grade and were used as received. All solutions were saturated with O_2 (Matheson). Unless stated otherwise, measurements were carried out at 290 nm. Scattered light measurements[6] were carried out for each of the enzyme concentrations used. The scattered light was less than 10% and was ignored.

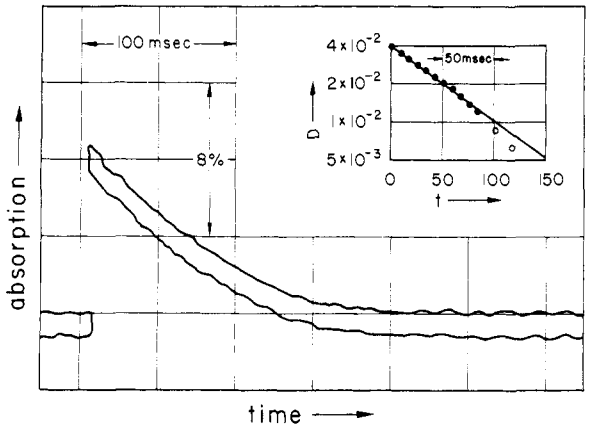

Figure 1. Process II. O_2 saturated solution of 10^{-2} M Na_2HPO_4, 10^{-3} M NaH_2PO_4, 10^{-2} M sodium formate, 4.8×10^{-8} M dismutase. $[O_2^-]_0 = 1.8 \times 10^{-5}$ M. pH 7.9. Half-life of O_2^- in the blank solutions was 200 msec. Measurements carried out at 290 nm.

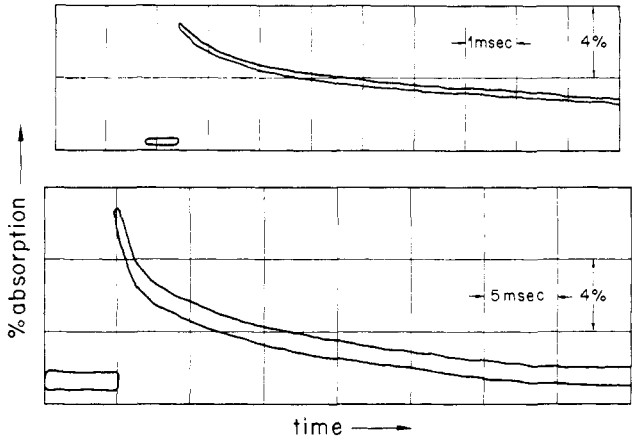

Figure 2. Oscilloscope traces showing both processes I and II. Measured at 280 nm in a 3.8×10^{-7} M dismutase solution; 1.8×10^{-5} M O_2^-. Other conditions as in Figure 1.

To minimize photolysis, the shutter between the lamp and the irradiation cell was opened just (~1 sec) before irradiation. We have found, however, that the opening of the shutter for 1 min before irradiation had no effect on the decay rate of O_2^-. Appropriate light filters were used to eliminate unnecessary light. Unless otherwise stated, a 4-cm single pass cell and a 150 W Xe-Hg lamp were used. The results represent the effects of single pulses on previously unirradiated solutions. Enzyme was stored up to 2 weeks in a solution saturated with $(NH_4)_2SO_4$. Ammonium sulfate was removed by dialysis 1 day before use. Oscilloscopes (Tektronix) (556 dual beam and 549 memory), a 1 P 28 photomultiplier, and a B and L monochromator were employed. The time resolution was less than 2 μsec, excluding the pulse duration (0.1–1.5 μsec).

The enzyme concentrations in the stock solutions were determined by the optical absorbances at 280 nm. An extinction coefficient 6.0×10^4 M^{-1} cm^{-1} was taken for the calculations of the concentrations. The pH of the solutions was found to be unaffected by the irradiation. The temperature was 23 ± 2°.

Results

Decay of Absorbance by Two Processes. An initial rise of absorbance at 290 nm due to the formation of O_2^- radical ions and its decay in the presence of 4.8×10^{-8} M dismutase are presented in Figure 1. As shown by the semilogarithmic plot, this decay is first order with respect to O_2^- for approximately 80% of the reaction, after which deviations are apparent. Such results are typical for total enzyme concentration $[E]_0 < 3 \times 10^{-7}$ M. The optical density in Figure 1 decays back to zero. At higher enzyme concentrations, residual optical densities remain for at least several hundred milliseconds. This will be presented later.

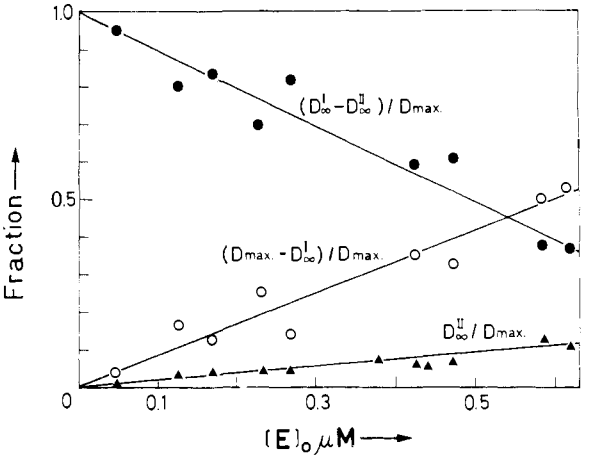

Figure 3. The effect of $[E]_0$ on processes I and II. pH 7.9 (phosphate buffer as in Figure 1); 1.8×10^{-5} M O_2^-. Measurements carried out at 290 nm. Each value is an average of 1–4 runs.

This process, which we shall call process II, was preceded by a more rapid decay whose extent increased with increasing $[E]_0$. Under the conditions used in Figure 1, the rapid initial decay could not have accounted for more than a few per cent of the total change, and therefore is not clearly seen in the figure. The faster decay process, at constant $[O_2^-]_0$, becomes more important at higher enzyme concentrations. Thus, at $[E]_0 = 4 \times 10^{-7}$ M, about 40% of the absorbance decays away before process II can be observed. This is demonstrated in Figure 2. We shall call the rapid initial decay of O_2^- process I. In Figure 3 we present data showing the relative contributions of process I and process II to the decay of absorbance at 290 nm. Under the conditions of Figure 3, the maximum optical density change, D_{max}, is equal within experimental error to the initial optical density of the O_2^- radical ions. D_∞^I and D_∞^{II} are the optical density changes at the end of processes I and II, respectively. The time separation between process I and process II was not good, as the rates of these two processes varied by a factor of about 10 only. (See Figure 2 for the separation between processes I and II.) Values of D_∞^I could be roughly determined as will be seen later.

There is a continuous decrease in the contribution of process II, and an increase in the contribution of process I, as $[E]_0$ increases from 0.05 to 0.7 μM. Although we have chosen to plot straight lines, the scatter of results (Figure 3) does not make it possible to attach special physical significance to the apparent linear dependencies.

Both processes I and II are characterized by apparent reaction rate constants "k_{II}" and "k_I," which are defined from eq 11. Plots of $\ln (D_t^I - D_\infty^I)$ *vs.* time were made lin-

$$\text{"}k_{II}\text{"} = (1/[E]_0)(d/dt) \ln (D_t^{II} - D_\infty^{II})$$
$$\text{"}k_I\text{"} = (1/[E]_0)(d/dt) \ln (D_t^I - D_\infty^I) \quad (11)$$

ear up to at least 90% reaction by an appropriate choice of D_∞^I. D_∞^I values, so determined, were similar to the D_∞^I values estimated intuitively from figures such as 2.

The apparent rate constants "k_I" and "k_{II}" were found to be independent of $[E]_0$. Namely, both processes I and II are first order in $[E]_0$. As will be seen later, similar "k_I" values were obtained at $[E]_0 > 0.7$ μM, where there was little or no interference from process II, and where the decay of absorbance was found to obey strictly a first-order rate law. Under such conditions, we found that process I was first order also in $[O_2^-]$.

Table I. Values of "k_I" and "k_{II}" [a]

$[E]_0$, μM	$[O_2^-]_0$, μM	pH[b]	"k_I" $\times 10^{-8}$, M^{-1} sec^{-1}	"k_{II}" $\times 10^{-8}$, M^{-1} sec^{-1}
0.050	18	7.9		1.6
0.050	8	7.9		1.5
0.050	18	6.9		2.3
0.083	18	7.9		1.3
0.13	18	7.9		1.5
0.13	18	7.4		1.3
0.13	8	7.4		2.0
0.17	18	7.9		1.3
0.24	18	6.8		1.9
0.24	18	7.9		1.9
0.38	18	7.8	20	2.0
0.38	8	7.8	15	
0.45	18	7.9	11	1.9
0.60	18	7.9	15	1.5

[a] Measured at 290 nm. Solutions contain 10 mM formate and 10^{-4} M EDTA. [b] pH 7.9 adjusted with 10^{-2} M Na_2HPO_4 + 10^{-3} M NaH_2PO_4. pH 7.4 adjusted with 10^{-2} M Na_2HPO_4 + 3 × 10^{-3} M NaH_2PO_4. pH 6.9 adjusted with 10^{-3} M Na_2HPO_4 + 10^{-3} M NaH_2PO_4.

In Table I we present "k_I" and "k_{II}" values measured under various conditions. The results show little, if any, dependency on pH in the range 6.9-7.9. Measurements at pH ~10 seem to give similar values of "k_{II}" but considerably lower (about threefold) "k_I."

The effect of changing initial $[O_2^-]$ at constant $[E]_0$ was investigated. Generally, decreasing $[O_2^-]_0$ had an effect similar to increasing $[E]_0$; namely, it resulted in a relative increase of the contribution of process I and of the residual absorption (D_∞^{II}), at the expense of process II. Thus, reducing $[O_2^-]_0$ from 1.8×10^{-5} to 9×10^{-6} M, in a 4×10^{-7} M dismutase solution (pH 7.8), decreased $(D_\infty^{I} - D_\infty^{II})/D_{max}$ from 0.52 to 0.20 and increased $(D_{max} - D_\infty^{I})/D_{max}$ and D_∞^{II}/D_{max} from 0.42 and 0.06 respectively to 0.72 and 0.08, respectively.

At relatively high $[E]_0$, process II is not observed. Process I, however, is still followed by a relatively small partial decay of absorbance (amounting to about 10% of the initial absorbance change). The half-life of this partial decay is about 10 msec, independent of $[E]_0$ above 1 μM. It will be referred to as "process II'." At 1 μM dismutase, process II is expected to have a lifetime of about 10 msec and cannot be separated from the $[E]_0$ independent process II'. We do not know the reason for process II'. It may be connected with some conformational changes in the enzyme which affect somewhat its optical absorption at 290 nm. The concentration of E at which process II disappears is a function of the initial $[O_2^-]$ present. At lower $[O_2^-]_0$ the elimination of process II takes place at lower $[E]_0$. The residual optical absorption, at the end of process II', decays back to zero after at least several seconds. The instability of our light source made it impossible to investigate quantitatively the last process. A possible role of photochemistry by the light source is still open to question.

In Table II we present "k_I" values measured under various conditions in solutions containing $[E]_0 > 0.7$ μM. Under these conditions, plots of ln $(D_t^{I} - D_\infty^{I})$ *vs.* time were usually linear up to at least 85% reaction. The values of "k_I" (Table II) are similar to those measured in low $[E]_0$ solutions (Table I). This shows that in both cases we observed the same process.

In order to test the possibility that low molecular weight impurities were responsible for the catalysis, an enzyme solution was divided into three parts: one part was kept for 16 hr without treatment; another part was dialyzed for 16 hr; the last part was heated to boiling in the presence of 10^{-4} M EDTA for a few minutes. The enzyme solutions were tested in the presence of 10^{-2} M formate, 10^{-4} M EDTA, at pH 7.9. The solutions which have not been boiled retained their full activity and gave results identical with those obtained in freshly prepared solutions. The solution which was boiled lost at least 97% of the activity. EDTA and catalase (10^{-9} M) did not have any effect on the results.

Table II. Values of "k_I" Measured in >0.7 μM Dismutase[a]

$[E]_0$, μM	$[O_2^-]_0$, μM	pH[b]	"k_I" $\times 10^{-9}$, M^{-1} sec^{-1}
0.77	20	6.8	2.0
1.4	5.0	7.9	1.9
1.5	19	7.9	1.6
2.3	20	6.8	1.2
2.4	19	7.9	1.2
2.4	7.6	7.9	1.4
2.4	3.0	7.9	1.1
2.4	19	7.9	0.9[c]
2.4	19	7.9	1.6[d]
4.7	19	7.9	1.7
7.1	19	7.9	1.2
9.4	19	7.9	1.7
12	1.2	7.9	1.3[e]

[a] See footnote *a* to Table I. [b] See footnote *b* to Table I. [c] 30 mM formate. [d] 3 mM formate. [e] Measured at 248 nm, 1 cm light path.

Discussion

Processes I and II. A Four-Step Mechanism. Our results are in agreement with a reaction sequence such as eq 12-15

$$E + O_2^- \longrightarrow E^- + O_2 \quad (12)$$

$$(\text{or } E + O_2^- \xrightarrow{2H^+} E^+ + H_2O_2)$$

$$E^- + O_2^- \xrightarrow{2H^+} E + H_2O_2 \quad (13)$$

$$(\text{or } E^+ + O_2^- \longrightarrow E + O_2)$$

$$E^- + O_2^- \longrightarrow E^{2-} + O_2 \quad (14)$$

$$(\text{or } E + O_2^- \longrightarrow E^- + O_2)$$

$$E^{2-} + O_2^- \xrightarrow{2H^+} E^- + H_2O_2 \quad (15)$$

$$(\text{or } E^- + O_2^- \xrightarrow{2H^+} E + H_2O_2)$$

between O_2^- and different forms of the enzyme. A similar reaction sequence has been previously proposed for Bovine Cu-Zn dismutase.[6] E^+, E, E^-, and E^{2-} may involve Mn^{IV}, Mn^{III}, Mn^{II}, and Mn^{I}, respectively.

The reaction rate constant $k_{12} = 1.3 \pm 0.15 \times 10^9$ M^{-1} sec^{-1} was measured at the relatively high $[E]_0/[O_2^-]_0 = 10$ (see Table II). We propose that at relatively high *catalytic* dismutase concentrations, where process II is absent, reactions 12, 13, and to some extent 14 are responsible for the decay of O_2^-. Reaction 15 is slow (this will be justified later) and can be neglected under the conditions of Table II. The average "k_I" which can be calculated from Table II is $1.5 \pm 0.1 \times 10^9$ M^{-1} sec^{-1} at pH 7.9, at constant 10^{-2} M formate. The apparent effect of [formate] on "k_I" is probably an ionic strength effect. (In the calculation of this "k_I" value we excluded the experiments at $[E]_0 = 12$ μM, since in these experiments the concentration of E was not catalytic.)

The absence of process II, the good first-order fits, and the independence of "k_I" on $[E]_0$ indicate that the accumulation of the relatively unreactive form of the enzyme was only little, under the conditions of Table II. Thus, had the product of reaction 14 accumulated up to 50% of $[E]_0$, a similar decrease of the O_2^- decay rate would have been ob-

served, contrary to the results of Table II. Therefore we feel justified to assume that reactions 12 and 13 do have the major contribution to O_2^- decay under the conditions of Table II. Using $k_{12} = 1.3 \times 10^9\ M^{-1}\ sec^{-1}$ and "k_I" $= 1.5 \times 10^9\ M^{-1}\ sec^{-1}$ (average of the experiments of Table II, pH 7.9, $10^{-2}\ M$ formate, in which the $[O_2^-]_0/[E]_0$ ratios were fairly low), we calculate $k_{13} = 1.6 \pm 0.6 \times 10^9\ M^{-1}\ sec^{-1}$. This value is based on $k_{14} = 2 \times 10^8\ M^{-1}\ sec^{-1}$ which we will justify later. In any case, the calculation of k_{12} is only little affected by k_{14}, provided $k_{13} \gg k_{14}$ as is indeed found. The calculation of k_{13} was carried out using a Hewlett-Packard calculator with a Schmidt type program.[16]

Now we turn to process II. A sufficient excess of $[O_2^-]_0$ over $[E]_0$ (low $[E]_0$ and high $[O_2^-]_0$; see Table I and Figures 1–3) is expected to convert E to a steady state mixture of all three forms of the enzyme, in which the less reactive form is predominant. Consequently, due to the inefficiency of reaction 15, the overall rate of O_2^- decay slows down, as is indeed observed during process II. It can be shown that under such steady state conditions, the apparent reaction rate constant "k_{II}" can be expressed in terms of the parameters $k_{12} - k_{15}$ (eq 16). This is in agreement with process

$$\text{"}k_{II}\text{"} = 2(k_{13} + k_{14})/(1 + k_{13}/k_{12} + k_{14}/k_{15}) \quad (16)$$

II being first order in both $[E]_0$ and $[O_2^-]$. However, we found that deviations from first-order time dependency occurred when both $[E]_0$ and $[O_2^-]$ were very low. The deviations were caused by a too fast decay of O_2^-, when its concentration decreased. This may perhaps be due to reaction −14, which is the reverse of reaction 14. If (−14) becomes

$$E^{2-} + O_2 \longrightarrow E^- + O_2^- \quad (-14)$$
$$(\text{or } E^- + O_2 \longrightarrow E + O_2^-)$$

important the steady state is shifted toward higher concentrations of the more active forms of the enzyme. Consequently, the more efficient reactions 12 and 13 have an increased contribution to the decay of O_2^- radical ions.

The average value of "k_{II}" (from Table I) is $1.6 \pm 0.25 \times 10^8\ M^{-1}\ sec^{-1}$ (pH 7.8–7.9). If the values of "k_{II}," k_{12}, and k_{13} are inserted in eq 16, it can be seen that if values of k_{14} are chosen in the range $0\text{–}10^9\ M^{-1}\ sec^{-1}$, the ratios k_{14}/k_{15} which can be calculated range from 2×10^1 to 3×10^1.

Preliminary computations, with an attempt to find the pair of k_{14} and k_{15} which gives the best fit with the data ("k_I," "k_{II}," and the fractions of absorbance decay during processes I and II), indicate that k_{14} is about $2 \times 10^8\ M^{-1}\ sec^{-1}$ and k_{15} is about $1 \times 10^7\ M^{-1}\ sec^{-1}$.

Our proposal is then, when only a slight (up to tenfold) excess of $[O_2^-]_0$ over $[E]_0$ is present, the catalysis takes place by reactions 12 and 13, with a considerably smaller contribution from reaction 14.

When $[O_2^-]_0/[E]_0$ is larger than 15, the fast process I still takes place in the beginning. However, an enzyme form with relatively low reactivity toward O_2^- accumulates after some time due to the slowness of reaction 15. When steady state is established, this form is the predominate form of the enzyme. The inefficiency of reaction 15 causes the catalysis to be relatively slow as indeed observed during process II. Since process I may consume not more than about 15 O_2^- radicals per each E molecule, which was initially present, a decrease of $[E]_0$ at constant $[O_2^-]_0$ is expected to decrease the fraction of O_2^- that decays away by process I and increase the fraction of O_2^- that decays away by process II. This is confirmed by the experiments (Figure 3). A similar effect is expected upon increasing $[O_2^-]_0$ at constant $[E]_0$, as is indeed observed.

Forman and Fridovich[7] have reported an apparent reaction rate constant $1.8 \times 10^9\ M^{-1}\ sec^{-1}$ for the *E. coli* Mn dismutase at pH 7.8. This value, based on competition work, is in good agreement with our "k_I" measured by pulse radiolysis.

Forman and Fridovich did not find evidence for the slower catalytic process II. Under their conditions, only low steady state concentrations of O_2^- are involved. Under such conditions, reactions 14 and 15 may be neglected even at low $[E]_0$, as the more active forms of the enzyme prevail, perhaps due to reaction −14. In any case, we have found that the regeneration of the active forms of the enzyme is relatively fast (of the order of seconds). This is in agreement with the enhanced O_2^- dismutation at low $[E]_0$ and low steady state concentrations of O_2^- as observed by Forman and Fridovich. The conditions used by Forman and Fridovich are closer to the natural conditions at which this dismutase operates, and the higher activity, measured by "k_I," describes the activity of the enzyme *in vivo*.

The *E. coli* manganese superoxide dismutase was originally reported to contain 1.8 atoms of Mn per molecule of enzyme,[15] but subsequent studies corrected this value to 1.2.[17] The optical and nmr properties of this enzyme indicate that the valence of the metal in the resting enzyme is Mn^{III}.[18] Since O_2^- possesses both oxidizing and reducing properties, reaction 12 may involve formation of either Mn^{II} or Mn^{IV} enzymes. If the enzyme contains actually two Mn atoms per molecule, then E^{2-} may be the enzyme with both atoms of manganese in the plus 2 valence state. If there is only one atom of manganese per molecule, as seems likely, one might propose that the conversion of E^- to E^{2-} involved the reduction of Mn^{II} to Mn^I, or alternatively the reduction of an unidentified component of this enzyme. It is not possible to conclude, on the basis of our results, which are the three states of the enzyme involved. Alternative mechanisms, similar to (12)-(15), but involving other oxidation states of the enzyme, are also possible. We have proved, however, that there are three forms of the enzyme, two of which are interconverted rapidly by O_2^-; a third is formed about 5 times more slowly and is considerably (about two orders of magnitude) less reactive as compared with the most efficient dismutase reactions.

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References and Notes

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Kinetic Studies of the Liquid Phase Peptide Synthesis

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Abstract: Kinetic studies were carried out on the newly developed "liquid-phase" method (LPM) for peptide synthesis, using soluble polymer esters of amino acids. The reaction rates of these polymer-bound amino acids (esters of polyethylene glycol with molecular weights in the range of 2,000 to 20,000) were compared with those of the low molecular weight components used in classical peptide coupling reactions. New techniques for evaluating spectroscopic data have enabled the peptide coupling reaction to be studied precisely. The reactions were shown to be of the second-order type. The comparison indicated that the LPM system showed analogous kinetic behavior to the system used for classic peptide synthesis. The reaction rates are of the same order of magnitude and both systems revealed linear kinetic behavior. Under otherwise identical conditions the presence of the polyethylene glycol esters caused an increase in reaction rate compared with the corresponding low molecular weight analogs. This was interpreted as being due to tautomeric catalysis.

The "solid-phase" method for peptide synthesis, introduced some 10 years ago, was initially considered a very promising and exciting new technique which gave rise to great hopes and expectations.[2a] It thus seemed that the automated synthesis of biologically important peptides or even proteins was within reach.[2b-4]

However, criticism of the method grew as the new technique became popular in many laboratories.[5,6] In addition to pure synthetic problems the utilization of an insoluble matrix and therefore heterogeneous reaction conditions causes new difficulties such as steric hindrance,[7,8] solvation[9] problems, etc. These problems were of such importance that they tended to question the general viability of this technique.

In order to solve these problems, we have recently developed the "liquid-phase" synthesis which basically consists of the following:[10,11] a soluble, linear homopolymer serves as the C-terminal protecting group for the peptide which is to be synthesized. Mono- or bifunctional polymers such as polyethylene glycol have proved themselves as being especially good. All reactions are carried out under homogeneous conditions but contrary to the classical method, the activated components can be used in large excess so that quantitative coupling is achieved. The yields from the individual coupling steps can then be determined using simple tests.

Two properties of the polymer enable the polymer-bound peptide to be separated from the low molecular weight material, namely: (a) its molecular weight, which means separation can be achieved *via* ultrafiltration; (b) and the polymer's tendency to crystallize which remains unaltered by the peptide chain.[12,13] Thus, by using this new method the difficulties of the "solid-phase" synthesis can be avoided and at the same time its positive aspects are preserved. In this paper we wish to report the effect of the macromolecular protecting group upon the coupling reaction rate and compare the latter with that encountered in classical peptide synthesis, where low molecular weight protecting groups are used. In order to study the reaction rates, exact kinetic data were necessary.

If one succeeds in reaching rate constants using the "liquid-phase" method which are equal to those in classical peptide synthesis, no additional problems caused by the polymer would disturb a peptide synthesis. Then only the chemical problems of peptide coupling have to be considered.

The coupling reaction using *p*-nitrophenyl esters has proved itself to be especially suitable for kinetic studies since the reaction can be followed easily spectroscopically. In order to detect and recognize slight differences in the kinetic behavior of various substrates used here, it was found necessary to develop new and accurate techniques which have been mentioned elsewhere.[14] *tert*-Butoxycarbonylglycine *p*-nitrophenyl ester was coupled to various glycine esters under identical reaction conditions whereby the high and low molecular weight C-terminal protecting group was varied. The glycine esters of the following alcohols were used: *tert*-butyl alcohol, ethanol, 2-methoxyethanol, and polyethylene glycol with the molecular weight 2,000, 4,000, 6,000, 10,000, and 20,000. A comparison between polyethylene glycol triglycine ester and the triglycine ethyl ester should show whether the results obtained in synthesizing the dipeptide can be applied to larger peptides.

Experimental Section

Materials. The following commercial products were used: acetonitrile (Uvasol Fluka and Uvasol Merck), further dried over molecular sieve (3 Å, Merck) and distilled; glycine *tert*-butyl ester hydrochloride (puriss, Fluka); glycine *tert*-butyl ester (puriss, Fluka); glycine ethyl ester hydrochloride (puriss, Fluka); triethylamine (puriss, Fluka); triglycine ethyl ester hydrochloride.[15] The commercial polyethylene glycols (Chemische Werke Hüls) were purified by ultrafiltration and by precipitation from solutions in methylene chloride with diethyl ether. The molecular weight distribution is less than ±7% of the molecular weights given.

Synthesis of Glycine Polyethylene Glycol Ester Hydrochloride with Various Molecular Weights. Polyethylene glycol (1 mmol) was dissolved in dry methylene chloride to form a 10% solution (w/v). BOC-Gly (1.75 g, 10 mmol) and DCC (2.06 g, 10 mmol) were added and the mixture was stirred at room temperature under anhydrous conditions for 6 days. The insoluble dicyclohexylurea was removed by filtration and the solvent removed *in vacuo*. The residue was then dissolved in 1.2 *N* HCl–acetic acid (10% solution, w/v) and stood at room temperature for 30 min to ensure complete cleavage of the protecting group.