STOPPED FLOW SPECTROPHOTOMETRIC OBSERVATION OF SUPEROXIDE DISMUTATION IN AQUEOUS SOLUTION*

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1. Introduction

There are few ways to develop a high concentration of superoxide in solutions of physiological significance, and by far the most useful of these, pulsed radiolysis of oxygenated solutions [1], requires very specialized and expensive research equipment. One of the long term goals of this laboratory has been to develop alternative procedures for examining the chemistry of superoxide ion in aqueous solution [2]. We now report some preliminary results obtained with a stopped-flow spectrophotometric system which mixes superoxide, stabilized in aprotic solvents, into aqueous solutions and allows direct photometric observation of the superoxide.

In this preliminary communication we present results on (a) the second-order dismutation of superoxide in the pH 7–11 range, (b) the kinetic behavior of bovine superoxide dismutase in this pH region, and (c) the decay of O_2^- in the presence of H_2O_2 which show that the Haber-Weiss [3] reaction

$$O_2^- + H_2O_2 \rightarrow OH\cdot + O_2 + OH^- \qquad (1)$$

is at most extremely inefficient compared to dismutation.

2. Materials and methods

Potassium superoxide was obtained from Alpha Inorganics, 96%, Lot #091874. 18-crown-6 polyether was purchased from PCR, Inc., Gainesville, Florida and spectrophotometric grade DMSO[1] was obtained from Aldrich Chemical Co. and used after drying over $CaSo_4$. Zn/Cu superoxide dismutase was isolated by a published procedure [4].

Solutions of superoxide in dimethylsulfoxide (DMSO) were prepared essentially as described by Valentine and Curtis [5]. The final concentration of 18-crown-6 was 40 mM and that of superoxide approximately 30 mM. The concentration of superoxide was determined by injecting a measured amount of the solution into 3 ml of 0.1 M potassium phosphate buffer, and oxygen release was monitored with a standardized Clark-type oxygen electrode (Yellow Springs Instrument Co.). The amount of oxygen released was taken as one-half the concentration of O_2^- in the DMSO according to

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

The amount of hydrogen peroxide formed was occasionally determined by subsequent addition of catalase which yielded an additional half stoichiometric increment of oxygen (cf. Ref. 2).

The stopped-flow instrument was of standard design [6], but a special mixer, which will be described at a later date, was required to obtain efficient mixing of DMSO and water. Briefly, we are mixing one part of DMSO with 25 parts of water by placing a ball mixer similar to that described by Berger et al. [7] in series with a standard Gibson [8] type mixer. The dead time [6] of the system under normal operating conditions was 5–6 msec. This system was sufficient to avoid the serious optical artefacts observed when

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DMSO is mixed with water in the usual multi-jet mixing arrangement [8]. The small optical change, $\lesssim 0.05\ \Delta A$, produced upon mixing DMSO and water was readily corrected for by either subtraction or minor adjustment of the final voltage obtained after reaction.

Superoxide was observed at 275 nm using an Osram 100 W tungsten-iodide lamp [9]. Excellent second-order decay plots were taken as evidence for lack of significant stray light. The photomultiplier output was read into a Nova 2/10 computer via an interface system supplied by On-Line Instrument Systems (OLIS) of Athens, Ga. Data collection and processing was carried out using software supplied by OLIS.

3. Results

The spontaneous and catalyzed dismutations of superoxide are shown in fig.1A and 1B, respectively. It is evident that spontaneous dismutation follows second order kinetics over a large fraction of the reaction. There is, however, significant deviation toward the latter stages of the reaction. As has been reported previously by several authors [9,10] the spontaneous dismutation consists of parallel, second and first order processes.

$$O_2^- + \text{catalyst} \rightarrow \qquad (3)$$

The integrated rate expression is

$$[O_2^-] = \frac{[O_2^-]_0\, e^{-k_3 t}}{\frac{2k_2}{\epsilon \ell k_3}\,[O_2^-]_0\,(1-e^{-k_3 t}) + 1} \qquad (4)$$

where k_3 is the first order rate constant, k_2 is the second order constant, ϵ is the molar absorptivity of O_2^-, ℓ is the pathlength of optical observation, and $[O_2^-]_0$ is the initial concentration of O_2^-. This expression has limits which approximate either a dominant second order decay or a dominant first order decay. Thus, when $2k_2/\epsilon\ell k_3 > 10$ and $[O_2^-]_0/\epsilon\ell \lesssim 1$ the initial slope of the plot of the reciprocal of absorbance vs time will give a good estimate of k_2, but at very long times a log plot will be linear and its slope will be the value of k_3. When the reaction is catalyzed by, for example, a dismutase the first order contribution can dominate ($k_3 > [O_2^-] \gg k_2$) and a plot of log absorbance against time will be linear with slope equal to k_3. These two extremes are illustrated in fig.1.

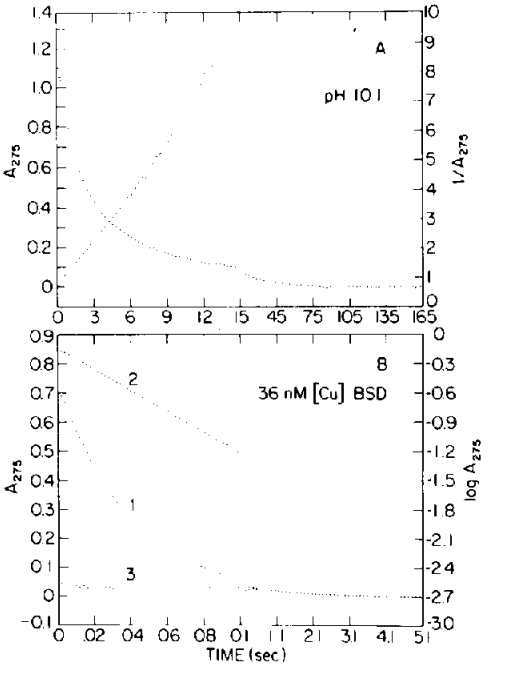

Fig.1. Demonstration of typical second-order (A), uncatalyzed, dismutation and first-order (B), catalyzed decay of superoxide by the stopped-flow spectrophotometric technique. The solution was 0.1 M potassium borate, 0.25 mM EDTA (pH 10.1) and in B contained 36 nM Cu as the bovine superoxide dismutase. The second order rate constant obtained from the initial portion of the $1/A_{275}$ trace using $\epsilon_{275} = 500\ M^{-1}\ cm^{-1}$ was 225 $M^{-1}\ sec^{-1}$. The catalytic rate constant, k_{cat}, obtained from the log A_{275} trace in B was $0.73 \times 10^9\ M^{-1}\ sec^{-1}$. $T \cong 22°C$.

It is well known that free metals such as Cu^{2+}, Mn^{2+}, and certain $Fe^{2+/3+}$ complexes are very effective catalysts of superoxide dismutation [11–13]. Relevant to this we have observed that the apparent rate of dismutation is decreased in all the buffers used, when EDTA was added to 0.1 mM, except pyrophosphate. The second order rate of dismutation was determined in several buffers over the pH range 7.2–11.3. Below pH 7 the reaction is too fast to observe with the present mixing efficiency. The error bars of fig.2 indicate an estimated precision of ± 5% and we subjectively

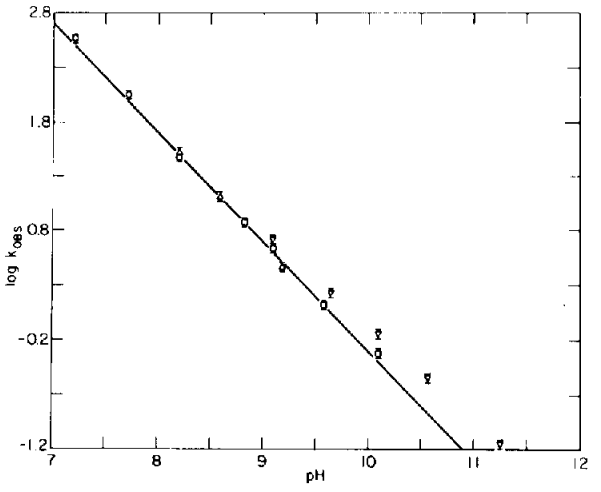

Fig.2. The second order rate constant for superoxide dismutation as a function of pH in the absence of added catalyst. Second order rate constants are plotted as the log of the observed rate of decay where $k_{obs} = 2k_2/\epsilon\ell$. θ 0.1 M potassium phosphate, △ 0.1 M sodium pyrophate, ▫ 0.1 M potassium borate, ▿ 0.1 M potassium glycinate, all buffers also contained 0.25 mM EDTA. Solid line is a theoretical fit as explained in the text. Error bars are for an estimated ± 5% precision error.

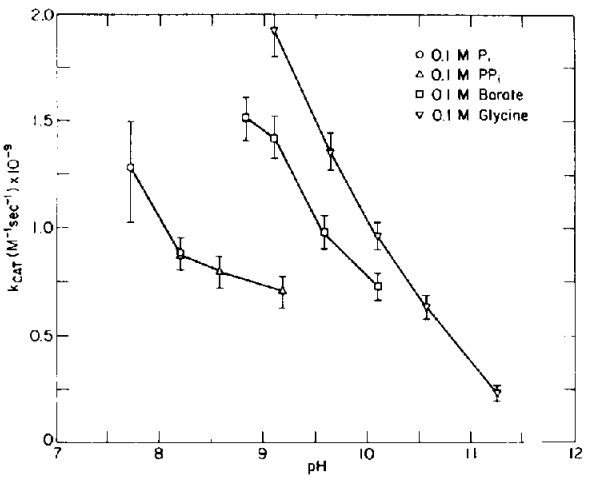

Fig.3. Catalytic rate constants, k_{cat}, for bovine superoxide dismutase as a function of pH and buffer. Conditions similar to those described in the legend to fig.1. k_{cat} was obtained by dividing the first order decay constant by the concentration of protein bound Cu.

estimate an accuracy of ± 20% for the reported constants.

Calculation of the second-order rate constant from optical changes requires a knowledge of the molar absorptivity of the species being observed. We have measured the concentration of O_2^- using an oxygen electrode as described above and also the initial absorbance due to O_2^- at high pH. Our best estimate of $\epsilon_{O_2^-}^{275}$ is 500 M^{-1} cm^{-1} with error limits of some 10%.

Figure 3 shows the catalytic rate constant of bovine superoxide dismutase as a function of pH in four buffer systems. It is evident that rate of the dismutation is subtly dependent on the nature of the buffer, and that there is a significant decrease in activity between pH 9 and 11 in glycine buffers.

Figure 4 shows the results of our attempts to determine the rate of the well-publicized [14–16] Haber-Weiss reaction. It can be seen that in two of the buffers, pyrophosphate and glycine, even 0.1 M H_2O_2 does not change the observed second-order decay constant of superoxide dismutation. In phosphate buffers a slight increase of the rate of dismutation in 0.1 M

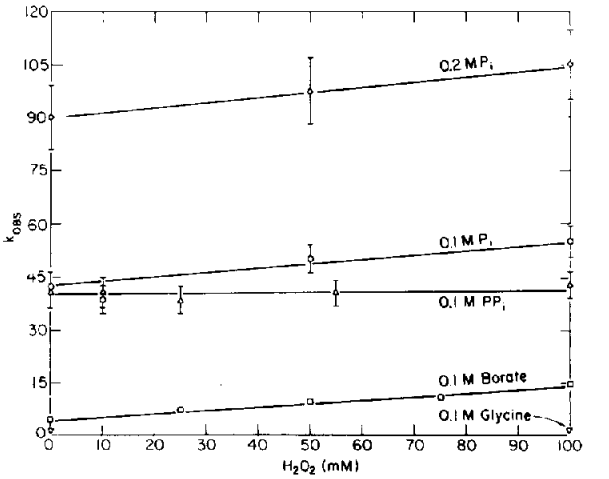

Fig.4. The effect of H_2O_2 on the observed second-order rate of superoxide dismutation in four buffers: ⊖, 0.2 M potassium phosphate (pH 8.2); ⊖, 0.1 M potassium phosphate (pH 8.2); ⏢, 0.1 M potassium pyrophosphate (pH 8.2); ⊟, 0.1 M potassium borate (pH 10.1); and ▾, 0.1 M glycine (KOH) (pH 10.6).

H_2O_2 was observed, but this is marginal being within our estimated error limits. The large contribution of the buffer to the second order rate constant should be noted. In borate buffer at pH 9.2 there appears to be a small increase of the dismutation rate with added peroxide which is outside the error limits. We do not believe this is due to reaction (1) but rather to an effect of H_2O_2 on the behavior of trace metal contaminants or on some other feature of the buffer leading to an increase in the second-order dismutation.

4. Discussion

The stopped-flow procedure appears to be an adequate tool for probing the chemistry of superoxide in aqueous solution. The second-order dismutation reaction is well defined and the presence of EDTA appears to minimize the first order contribution observed by many other workers [10]. In the presence of a catalyst such as a superoxide dismutate the expected crossover the first-order kinetics was observed.

The rate constants presented in fig.2 follow the rate law

$$k_{obs} = 2\left(\frac{k_6[H^+]}{K_{HO_2}} + k_4\right) \quad (5)$$

obtained from

$$O_2^- + HO_2 \rightarrow HO_2^- + O_2 \quad (6)$$

$$2\,O_2^- \rightarrow O_2 + O_2^{2-} (= H_2O_2) \quad (7)$$

and

$$K_{HO_2} = \frac{[H^+]\,[O_2^-]}{[HO_2]} = 10^{-4.88}\ (17). \quad (8)$$

From this expression $k_7 < 10\ M^{-1}\ sec^{-1}$ even for the glycine buffers and $k_6 = 3.1 \times 10^7\ M^{-1}\ sec^{-1}$ using $\epsilon_{O_2^-}^{275} = 500\ M^{-1}\ cm^{-1}$. Rabani and Neilsen [9] obtained a value of $k_6 = 7.7 \times 10^7\ M^{-1}\ sec^{-1}$ using $\epsilon_{O_2^-}^{275} \cong 1100\ M^{-1}\ cm^{-1}$ (cf. their fig.1). The approximate factor of two between the data sets is thus due in part to the different absorptivities used. At present there is some controversy over the spectral properties of O_2^- and HO_2, Bielski and Gebicki [17], suggesting that $\epsilon_{O_2^-}^{240} = 1050\ M^{-1}\ m^{-1}$ is correct while Czapski [18] maintains that $1950\ M^{-1}\ cm^{-1}$ is the better value Our present result would tend to agree with Bielski and Gebicki [17] but the value, $\epsilon_{250} = 2.6 \times 10^3\ M^{-1}\ sec^{-1}$, reported previously [2] for O_2^- in acetonitrile is close to that favored by Czapski [18].

The deviation of the rate constants (fig.2) from Equation 5 may be due to a small contribution from Reaction 7 but we estimate $k_7 \lesssim 10\ M^{-1}\ sec^{-1}$ in the borate buffers in good agreement with $\sim 6\ M^{-1}\ sec^{-1}$ reported by Divisek and Kastening [19]. The greater deviation in glycine buffer may be due to a real catalytic effect of the buffer.

The data of fig.3 demonstrates the usefulness of the technique in obtaining the catalytic rate constants for superoxide dismutases. The values presented are based on copper concentration and are comparable for those reported by other workers [20,21]. The small specific buffer effects are not understood, but these could contribute in part to difficulty in comparing specific activities in different buffers. The loss of activity between pH 9 and 11 is also comparable to results reported by others [22,23]. This may be due to ionization [24] of the water molecule bound to the Cu^{2+} [25] or Cu^+ [26].

Reaction (1) has often been used to 'explain' why both catalase and superoxide dismutase can influence a reaction or process involving molecular oxygen. This in spite of the fact that there exists a significant body of literature demonstrating that (1) is at most a very inefficient process (cf. Refs. 27 and 28). The data of fig.4 confirms the earlier conclusion of other chemists which has been ignored for the past several years.

Acknowledgments

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