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A Consideration of the Effects of Added Solutes on the Activity of Bovine Superoxide Dismutase

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Pulse radiolysis was used to study the effects of changing the concentration of buffer and added salts on the activity of bovine superoxide dismutase. The results are discussed with reference to other work and are interpreted mainly in terms of ionic-strength effects. The effects are best considered as empirical parameters, which are essential for comparative activity measurements. The specific activity of the enzyme was higher than found in previous pulse-radiolysis studies.

Bovine erythrocyte (EC 1.15.1.1), a Cu-(copper-containing) superoxide dismutase, has been widely studied since it was first isolated (for reviews see Fridovich, 1974, 1975). For the comparison of activities of different samples of enzyme there is as yet no universally used convention. The effects of added solutes on the activity of Cu-superoxide dismutase have been considered by Rigo *et al.* (1975), using polarography, and by McClune & Fee (1976), using stopped-flow spectrophotometry. Pulse radiolysis is the most useful method of studying $O_2^{\cdot -}$ dismutation (see, for example, Fielden *et al.*, 1974), and in the course of investigations on the properties of bovine erythrocyte the effects of certain added solutes on enzyme activity were measured. Particular attention was paid to the effect of added formate, which is frequently used (instead of ethanol) to convert hydroxyl radicals into $O_2^{\cdot -}$ in irradiated aerated solutions, and to the effect of pyrophosphate and borate, which are useful buffers at pH 9, where spontaneous dismutation of $O_2^{\cdot -}$ is slow compared with enzyme-catalysed decay.

In view of the work of Rigo *et al.* (1975) and McClune & Fee (1976) the results obtained by using pulse radiolysis deserve discussion.

Materials and Methods

Cu-superoxide dismutase was isolated from bovine erythrocytes by the method of McCord & Fridovich (1969) and protein concentrations were determined by using $\epsilon_{280} = 300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Enzyme activities were measured in air-saturated media by using the standard pulse-radiolysis technique (Fielden *et al.*, 1974). The assays were carried out by using approx. $0.02 \mu\text{M}$ -superoxide dismutase and $15 \mu\text{M}$ - $O_2^{\cdot -}$. A transient recorder enabled the data points from the exponential-decay traces to be fed directly into a computer, and subsequently a catalytic second-order

rate constant was calculated by using a least-squares method.

All solutions were prepared from analytical-grade chemicals dissolved in triple-distilled water, and pH measurements were made immediately before irradiation. In view of the low buffer concentrations, effective buffering was obtained by working near the pK values of the buffers at 25°C. In the presence of 0.5 mM-sodium pyrophosphate the enzyme activity was unchanged between pH 8.95 and 9.25.

EDTA ($100 \mu\text{M}$) was present in all solutions and its small contribution to the ionic strength (I) was considered in calculating the total value of I , although the exact species in solution are not known if the EDTA serves to complex any adventitious metal ions. In 0.35 mM-sodium borate buffer containing 0.1 M-ethanol the enzyme activity was unchanged for concentrations of EDTA between 25 and $100 \mu\text{M}$. In the same buffer containing $100 \mu\text{M}$ -EDTA variation of the ethanol concentration (0.05–0.1 M) did not change the enzymic activity.

The second-order rate constant chosen as a reference [$k_{\text{ref.}} = (3.70 \pm 0.18) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ s.d., $n=9$)] refers to a medium containing 0.5 mM-sodium pyrophosphate, $100 \mu\text{M}$ -EDTA and 0.1 M-ethanol. Assay under such conditions was performed during each set of experiments. The more conventional choice of a reference as k_0 (i.e. the rate constant for $I=0$) was not possible because extrapolation to zero ionic strength is invalid (see below). In all cases the effects of added salts (including buffers) on the activity are presented as plots of $\log(k/k_{\text{ref.}})$ against $I^{\frac{1}{2}}$ (Figs. 1, 2 and 3).

Results and Discussion

The Brönsted-Bjerrum equation (Brönsted, 1922, 1925; Bjerrum, 1924, 1925) referring to the reaction between two charged species in dilute

Fig. 1. Variation of superoxide dismutase activity with buffer concentration

The effect on the second-order catalytic rate constant of added sodium pyrophosphate ($\text{pH}8.95 \pm 0.05$) (○) and sodium tetraborate ($\text{pH}9.25 \pm 0.05$) (Δ) is shown. The medium contained 0.1M-ethanol and 100 μM -EDTA.

Fig. 2. Effect of Na_2SO_4 on the superoxide dismutase activity

The effect on the second-order catalytic rate constant of added Na_2SO_4 in the presence of 0.35M-sodium borate ($\text{pH}9.25 \pm 0.05$) (○) or 2M-sodium pyrophosphate ($\text{pH}8.95 \pm 0.05$) (●) is shown. The medium contained 0.1M-ethanol and 100 μM -EDTA.

solution shows that a plot of the logarithm of the second-order rate constant (k) against I^* should be linear with a slope proportional to the product of the charges of the two species. This correlation is valid only in solutions of ionic strength less than about 0.01M (see, for example, Frost & Pearson, 1961). It is often found that at higher ionic strengths the linearity of the plots is maintained, but the gradients of the lines in this region should not be used in conjunction with the Brönsted-Bjerrum equation (Frost & Pearson, 1961). Thus the mechanistic deductions of Rigo *et al.* (1975), from their measurements made only at high ionic strengths, would not

Fig. 3. Effect of NaCl and of sodium formate on the superoxide dismutase activity

The effect on the second-order catalytic rate constant of added NaCl in the presence of 0.35M-borate/0.1M-ethanol ($\text{pH}9.25 \pm 0.05$) (Δ) and of sodium formate in the presence of 2M-pyrophosphate (○) or in the presence of 0.5M-pyrophosphate at $\text{pH}8.95 \pm 0.05$ (●) are shown. The medium contained 100 μM -EDTA.

be expected to be valid. In the present work, for $I^* > 0.1$, the enzyme activity shows a slightly smaller dependence on I^* (Figs. 1, 2 and 3) than that reported by Rigo *et al.* (1975), but, for these ionic strengths, there is broad agreement between the two sets of results. However, it is clear that in the region where the Brönsted-Bjerrum relationship is valid for simple ions there is very little dependence of enzyme activity on ionic strength (Figs. 1, 2 and 3). The discontinuity in the ionic-strength-dependence of the activity stresses the need to study such effects over the correct range of electrolyte concentration, at least if mechanistic conclusions are to be considered.

Even if observations are made over the ionic strength region where the Brönsted-Bjerrum relationship is valid, it must be remembered that in the present case we are considering an enzyme-substrate interaction and not an interaction between two simple ions. The problem is no longer one of charge distribution with spherical symmetry, as is implicit in the Brönsted-Bjerrum approximation, and the specific nature of interaction at the protein surface must be considered (Alberty & Hammes, 1958). The problem of non-spherical charge distribution in the reaction of charged substrates with enzyme molecules, with particular reference to diffusion-controlled reactions, and the validity of the Brönsted-Bjerrum equation in such systems, has been discussed (Kuo-Chen *et al.*, 1975). The predictions suggest that deviations in linearity of a Brönsted-Bjerrum plot may occur at ionic strengths even lower than those expected for the reactions between simple ions.

The reason that there is little variation of activity with ionic strength for $I^{\frac{1}{2}} < 0.10$ is not understood, although in view of the results mentioned in the previous paragraph it would be unwise to interpret them by using simply the Brönsted-Bjerrum relationship. However, at high ionic strengths, the similar observations made with different solutes suggests a major contribution from general coulombic effects. The effects of ionic strength may be due to changes in the protein structure as well as to changes in the direct forces of interaction between substrate and enzyme. Thus the results are best regarded as useful empirical parameters.

McClune & Fee (1976) report that the activity of bovine erythrocyte cytochrome *c* at a fixed pH is dependent on the buffer present in the medium. These variations may be explained, at least partly, in terms of ionic-strength effects rather than just specific buffer effects as proposed. Similarly, their observed variations of activity with pH in a given medium may also be partly due to changes in ionic strength; at the concentration of buffer used (0.1M) such changes will be large, especially near the pK of the buffer. It is clearly necessary to consider any general effects of ionic strength before a discussion of possible specific solute effects.

The specific activity of the enzyme isolation used in the present work can be compared with those of the isolations used in previous pulse-radiolytic studies. Klug *et al.* (1972) reported a second-order catalytic rate constant of about $2.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 8–9 in a medium containing 0.01M-sodium formate. At a similar ionic strength a value of $(3.70 \pm 0.18) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ is found in the present work. Fielden *et al.* (1974) reported a second-order rate constant of $2.37 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ in a medium containing 2mM-sodium pyrophosphate and 0.085M-ethanol at pH 9.0–9.5. In a similar medium of comparable ionic strength (Fig. 1) the rate constant found in the present study was $(3.30 \pm 0.16) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. It is clear that the enzyme isolation used here has a considerably higher specific activity than those of previous pulse-radiolytic studies. It would be of interest to repeat the mechanistic experiments described by Fielden *et al.* (1974) except using

isolations of dismutase with higher specific activities to check whether their mechanism still applies.

Conclusions

The effects of the selected added solutes on the activity of bovine superoxide dismutase are probably ionic-strength effects. The biphasic nature of the Brönsted-Bjerrum plots emphasizes that at high ionic strengths it is invalid to use the gradients of such plots as criteria of mechanism. Previously reported variations of activity with pH and buffer composition are likely to be partly due to general effects of ionic strength. Assay media used in the evaluation of the activity of different isolations of bovine superoxide dismutase must be of the same composition if comparisons are to be valid.

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