Comparative kinetic study between native and chemically modified Cu,Zn superoxide dismutases

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The kinetic behaviour of native bovine erythrocyte Cu,Zn superoxide dismutase (N-SOD) and of its derivatives by reaction with polyethylene glycol, acetic and succinic anhydrides has been investigated here in detail. Their responses to changes of pH and ionic strength (*I*) have been used as a probe for quantitatively displaying the relevance to kinetic rate constant of superficial positive charges driving the superoxide ion (O_2^-) toward the enzyme's active site. Overall kinetic trends indicate that this long-range O_2^- electrostatic guidance is essentially due to the positive charges of the amino-acid residues Lys-120 and Lys-134 which are strategically located around the active site. The comparison between the kinetic data obtained from N-SOD and those from polyethylene-glycolated SOD (PEG-SOD) enabled us

to state that in PEG-SOD an O_2^- -steering positive electrostatic force, halved in comparison with N-SOD, is still operating, and that only Lys-120 is linked in the reaction of N-SOD with PEG. Elimination of the electrostatic driving force, carried out either by deprotonation of lysine amino groups at high pH, or by their neutralization with succinic anhydride and acetic anhydride, or by ionic screening at high ionic strength, always lowered the kinetic rate constant to a value of approx. $3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value is about 15 times smaller than that measured in the presence of the reactant-steering mechanism and represents the *k* value of the reaction limited by pure diffusion. Finally, the kinetic behaviour of acetylated SOD and succinylated SOD demonstrated the inhibitor effect of OH^- at strongly alkaline pH.

INTRODUCTION

Native bovine erythrocyte Cu,Zn superoxide dismutase (N-SOD) is a homodimeric enzyme, each subunit containing a catalytically essential copper(II) ion bridged by a histidine residue to a solvent-inaccessible zinc(II) ion (Tainer et al., 1982).

This enzyme catalyses very effectively the O_2^- dismutation into O_2 and H_2O_2 (McCord and Fridovich, 1969) by a cyclic oxidation–reduction mechanism, according to the conventional kinetic scheme (Klug-Roth et al., 1973; Fielden et al., 1974):

The reactions of O_2^- with either the reduced or oxidized form of the enzyme have extremely high and equal rate constants, $k_{+1} = k_{+2} = k$, of the order of $2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Klug-Roth et al., 1973; Fielden et al., 1974; Viglino et al., 1981).

These peculiar kinetic features suggested that the rate-determining step of the enzymic reaction was the reactant-encounter rate (Cudd and Fridovich, 1982), except under particular conditions (Fee and Bull, 1986).

In fact, the catalytic mechanism could not be explained simply on the basis of the chemical properties of the active-site copper. First, they could not justify the equality between k_{+1} and k_{+2} , owing to the difference in reactivity of copper in the oxidized and reduced forms. Secondly, they do not account for the kinetic constant value (*k*), which is much higher than that calculated for the pure diffusion-controlled reaction (Koppenol, 1981; Sharp et al., 1987).

Consequently, an electrostatic guidance facilitating the encounter of the substrate with the active site has been suggested (Koppenol, 1981; Cudd and Fridovich, 1982).

This has been inferred by the experimental data also relevant to the influence of salt concentration on *k*.

For the reactions between like-charged ions, simple diffusion models predict an increase of *k* with increasing *I*. Such behaviour should be expected for the N-SOD/ O_2^- reaction, given that N-SOD, which has an isoelectric point (pI) of 5.2 (Bannister et al., 1971), at pH values higher than 5.2 is negatively charged, as indeed is the superoxide ion.

Conversely, a decrease of *k* with increasing *I* has been found by many authors (Rigo et al., 1975; Cudd and Fridovich, 1982; Argese et al., 1987; O'Neill et al., 1988). This behaviour was explained by the presence of an asymmetrical charge distribution on the enzyme surface, with a prevalence of positive charges around the active site, providing an electrostatic guidance of O_2^- towards the copper site (Koppenol, 1981; Getzoff et al., 1983).

On the basis of chemical and structural studies (Cudd and Fridovich, 1982; Tainer et al., 1982; Getzoff et al., 1983) and of recent theoretical models (Allison et al., 1985; Sharp et al., 1987; Bacquet et al., 1988), it has been proposed that the groups responsible for electrostatic guidance were the positively charged ϵ -amino groups of Lys-120, Lys-134 and the guanidinium group of Arg-141 (Head-Gordon and Brooks, 1987; Allison et al., 1988; Sines et al., 1990).

Indeed, elimination of the positive charges on the lysine residues (caused either by deprotonation at high pH, by acetylation, or by succinylation) involves a considerable decrease of *k* and of the *I* effect (Cudd and Fridovich, 1982; Argese et al., 1987). Furthermore, modification of Arg-141 causes a drastic loss of activity (Malinowski and Fridovich, 1979; Cocco et al., 1982; Borders et al., 1985; Beyer et al., 1987; Banci et al., 1988).

The aim of this work has been to provide additional evidence for the reactant-steering mechanism of N-SOD and to acquire an accurate picture of the kinetic behaviour of acetylated SOD

Abbreviations used: SOD, bovine erythrocyte Cu,Zn superoxide dismutase; E-Cu²⁺, oxidized form of the active site of SOD; E-Cu⁺, reduced form of the active site of SOD; N-SOD, native SOD; PEG-SOD, polyethylene-glycolated SOD; SUCC-SOD, succinylated SOD; ACE-SOD, acetylated SOD; PEG, polyethylene glycol; Z_o, charge experienced by superoxide ion approaching the active site of the enzyme.

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(ACE-SOD), succinylated SOD (SUCC-SOD) and particularly of polyethylene-glycolated SOD (PEG-SOD), a SOD derivative which has been shown to have interesting applications in biochemical and biomedical fields.

MATERIALS AND METHODS

All the solutions were prepared from analytical grade chemicals in water purified using a Millipore Milli-RO Milli-Q system. Acetic anhydride, succinic anhydride, SOD and polyethylene-glycol-modified SOD were obtained from Sigma (St. Louis, MO, U.S.A.).

Enzyme activity was measured in air-saturated solutions by a modified polarographic method of catalytic currents (Argese et al., 1984). Polarographic waves were collected by an Amel 470 multipolarograph equipped with a Linseis L-800 xy recorder. Tris(hydroxymethyl)aminomethane perchlorate, sodium borate and *n*-propylamine perchlorate buffers were used in the pH ranges 7.7–8.5, 8.9–10.1 and 10.5–11.7 respectively. A 0.02 M NaOH solution was employed at pH 12.3. All buffer solutions had a starting *I* value of 0.02 M. This was increased by the addition of controlled amounts of NaClO₄ inert salt (Calabrese et al., 1983). All measurements were carried out at 22 °C. Activity values were found to be independent of the type of buffer used to regulate the pH.

For each enzyme the concentration was calculated, after dialysis, on the basis of the amount of Cu found using a Perkin–Elmer Model 5000 atomic absorption photometer equipped with a graphite furnace. Enzyme concentrations were also checked through electronic spectroscopy at 680 nm, using an ϵ value of 310 M⁻¹·cm⁻¹ (Pantoliano et al., 1982), by a Hewlett–Packard 8671A spectrophotometer.

Succinylation and acetylation were performed essentially according to Cudd and Fridovich (1982). SOD was dissolved in 50 mM potassium phosphate at pH 7.8 and 4 °C to give a final protein concentration of 0.1 mM. Anhydride was then added, little by little with continuous stirring, to a concentration of 200 mM. During the course of the reaction the pH of the mixture was maintained around 8.0 by the addition of 1 M NaOH. The solution was then stirred at 4 °C for 1 h. Reagents were removed by dialysis against 10 mM potassium phosphate at pH 7.8.

RESULTS AND DISCUSSION

Comparison between kinetic behaviours of N-SOD and PEG-SOD

The kinetic constant values of N-SOD and PEG-SOD in the ranges of pH 7.7–12.3 and of *I* 0.02–0.25 M are reported in Figures 1 and 2 respectively.

The response of N-SOD to pH and *I* changes corresponds very closely to that which has been reported in previous work (Argese et al., 1987) and is presented here for comparison and discussion purposes.

For both enzymes, an increase of *I* entails a concomitant activity decrease, as expected for a diffusion-controlled reaction between oppositely charged species. Therefore the *I* effect shows that O₂⁻ experiences a positive potential as it approaches the active site of both enzymes.

Both proteins carry a negative total charge, N-SOD and PEG-SOD having isoelectric points of 5.2 (Bannister et al., 1971) and 4.4 (McGoff et al., 1988) respectively. Thus the above trends can be explained only by an asymmetrical charge distribution on both the enzymes, with a prevalence of positively charged groups around the active site. These have been identified in the

protonated amino groups of Lys-120, Lys-134 and Arg-141 located at 1.2 nm (12 Å), 1.3 nm (13 Å) and 0.5 nm (5 Å) respectively from the active-site copper (Tainer et al., 1982; Getzthoff et al., 1983).

According to the above-mentioned hypotheses, the decreases in *k* at pH values > 9.3 (see Figures 1 and 2) should be ascribed to the gradual deprotonation of these positively charged groups that eliminates the O₂⁻-steering electrostatic field.

The sigmoidal trends of *k* values versus pH at different *I* values, as reported in Figures 1 and 2, are simulated by titration curves of the amino groups of the lysines (Argese et al., 1987), with mean p*K*_a values of about 10.6 for N-SOD and 10.9 for PEG-SOD (curves of Figures 1 and 2).

The mean value calculated for N-SOD agrees with the values of 10.1 and 10.8 found for Lys-120 and Lys-134 respectively (Argese et al., 1987).

As shown in Figures 1 and 2, all *k* values at increasing values of pH and *I* tend to converge to the value of about 3 × 10⁸ M⁻¹·s⁻¹ measured at pH 12.3.

At this pH value, the positively charged groups responsible for long-range electrostatic guidance of O₂⁻ appear to be completely neutralized, and hence the *k* value is determined by pure diffusion and by chemical properties of the active site.

The convergence of the *k* values and the assessed p*K*_a values suggest that the long-range O₂⁻ guidance arises mainly from the lysine residue charges located near the active site.

Conversely, Arg-141, the guanidinium group of which has a p*K*_a of 12.5 (Lehninger, 1970), does not appear to significantly contribute to long-range steering of O₂⁻ (Beyer et al., 1987).

In spite of the similar catalytic mechanisms, N-SOD and PEG-SOD present kinetic differences, as can be seen from Figures 1 and 2.

The treatment of N-SOD with PEG transforms the amino groups of some superficial lysine residues into neutral uretanic groups. Therefore the positive charges associated with them disappear (Veronese et al., 1985) and a consequent reduction of enzyme activity occurs.

Figure 1 Dependence of kinetic rate constant (*k*) of N-SOD on pH at various ionic strength (*I*) values

The experiments were carried out in solutions saturated with triphenylphosphine oxide (Argese et al., 1984). The lines represent the best-fit curves calculated by taking into consideration a single mean p*K*_a. *I* was increased with NaClO₄. Buffers employed are described in the Materials and methods. *I* = ●, 0.02 M; ■, 0.04 M; ▲, 0.09 M; ○, 0.16 M; □, 0.25 M.

Figure 2 Dependence of kinetic rate constant of PEG-SOD on pH at various ionic strength (I) values

$I = \bullet, 0.02 \text{ M}; \blacksquare, 0.04 \text{ M}; \blacktriangle, 0.09 \text{ M}; \circ, 0.16 \text{ M}; \square, 0.25 \text{ M}$. Experimental conditions and meaning of lines were as given in Figure 1.

In fact, at an I value of 0.02 M and a pH value of 9.3 the experimental data reported in Figures 1 and 2 display a k value for PEG-SOD that is 40% less than that for N-SOD. Furthermore, the salt effect is smaller for PEG-SOD than for N-SOD. For example, the activity decrease at pH 8.1 , with I increasing from 0.02 to 0.25 , is 50% for PEG-SOD and 70% for N-SOD.

For both N-SOD and PEG-SOD the trends of k versus I appear to be in agreement with the ones predicted by Alberty and Hammes (Alberty and Hammes, 1958; Hammes and Alberty, 1959; Argese et al., 1987).

They applied the theory of diffusion-controlled reactions to the case of the interaction between a charged low-molecular-mass substrate and the active site of an enzyme, obtaining the equation:

$$\log k = A Z_e Z_s \sqrt{I} + \log k_0 \quad (3)$$

in which A is a constant with a value of about $1 \text{ M}^{-\frac{1}{2}}$ at 22°C ; k_0 is the kinetic constant extrapolated to zero ionic strength; Z_s is the substrate charge and Z_e is an electrostatic factor virtually denoting the effective charge experienced by the substrate approaching the active site and quantitatively related to the potential gradient focusing O_2^- .

Figure 3 reports the straight lines obtained for PEG-SOD by plotting $\log k$ versus $\sqrt{I}$ at different pH values.

In Figure 4 the trends of Z_e versus pH for N-SOD and PEG-SOD are shown. The Z_e values were calculated from the slopes of $\log k$ versus $\sqrt{I}$ linear plots.

For both enzymes, the trends of Z_e versus pH shown in Figure 4 are very similar to the sigmoidal profiles of k versus pH of Figures 1 and 2, and are likewise simulated by titration curves of amino groups with pK_a values of 10.6 for N-SOD and 10.9 for PEG-SOD. This constitutes a good verification of the essential role played by the positively charged lysine amino groups in controlling the catalytic activity of both enzymes. It shows that eventual conformational modifications of the proteins due to pH and I changes affect the activity negligibly.

As is evident from the comparison of the curves of Figure 4, a PEG-SOD virtual charge of about half that present in the native

Figure 3 Influence of ionic strength (I) on the kinetic rate constant for interaction of O_2^- with PEG-SOD

pH = $\triangle, 7.7; \bullet, 10.5; \square, 11.0; \blacktriangle, 11.4; \circ, 12.3$. Experimental conditions were as given in Figure 1.

Figure 4 Plot of Z_e versus pH for N-SOD and PEG-SOD

The values of charge number Z_e were calculated according to eqn. 3 from the experimental data of Figures 1 and 2. $\circ$, N-SOD; $\bullet$, PEG-SOD.

enzyme is operating at pH values < 9 : this entails an activity loss of about 40% .

The PEG reaction does not cause significant structural changes of the active site (McGoff et al., 1988; Banci et al., 1990). From our results, in agreement with the results of Veronese et al. (1985), it is possible to attribute the PEG-SOD minor activity and the concomitant charge-halving to the modification of only one of the lysine residues responsible for O_2^- -guidance.

Indeed, it is necessary to point out that the reaction of SOD with PEG entails an activity loss which is never lower than a limit value; Veronese et al. (1985) measured, after extensive chemical modification with PEG, a residual activity of about 70% , which is near to the 60% we found using different methods and assay conditions.

This indicates that Lys-120 and Lys-134 are not equally reactive with respect to PEG and are not of equal weight with respect to the electrostatic guidance. In particular, the pK_a value of 10.9, obtained from the curves of k versus pH and Z_e versus pH for PEG-SOD, supports the hypothesis that the lysine residue reacting with PEG is the one having a pK_a value of 10.1 (Argese et al., 1987).

Finally, it is noticeable that at a pH value of 12.3, the kinetic constants of N-SOD and of PEG-SOD appear to be almost equal ($2.9 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $2.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively, see Figures 1 and 2).

This is consistent with the statement that the active site is not structurally affected by the PEG reaction (Banci et al., 1990). In fact, the driving potential is wholly eliminated at this pH by complete deprotonation of the lysine residue not linked with PEG. Thus the enzymic activity is controlled only by the diffusion and the structural features of the active site.

Kinetic behaviour of SUCC-SOD

The reaction with succinic anhydride transforms the positively charged amino groups of lysine residues into succinamidic groups carrying a negative charge, without altering the active site (Marmocchi et al., 1982; Cocco et al., 1983). This is greatly reflected either by the k value or by its dependence on pH and I , as is displayed by the trends reported in Figure 5.

Unlike N-SOD and PEG-SOD, the kinetic constant of SUCC-SOD increases with increasing I . Furthermore, after the reaction with succinic anhydride a drastic loss of enzymic activity was found (at pH values < 9 and at $I = 0.02 \text{ M}$ the k value is about 5% of that for native enzyme).

These findings further support the O_2^- electrostatic-guidance statement. They can be explained only if one admits that the charge inversion by succinylation replaces the attractor electrostatic field with a field opposing the O_2^- approach.

Figure 5 shows that the k value of SUCC-SOD is constant at pH values < 10.5 , consistent with the complete deprotonation of the carboxylic groups in the overall pH range assayed. As pH increases above the 10.5 value, the activity progressively decreases. This can be ascribed either to the OH^- anion which, by

Figure 5 Dependence of kinetic rate constant of SUCC-SOD on pH at various ionic strength (I) values

Experimental conditions and meaning of lines were as given in Figure 1. I values: ●, 0.02 M; ■, 0.04 M; ▲, 0.09 M; ○, 0.16 M; □, 0.25 M.

Figure 6 Dependence of kinetic rate constant of ACE-SOD on pH at various ionic strength (I) values

I values: ■, 0.04 M; ○, 0.16 M; □, 0.25 M. Results corresponding to other I strength values are not shown for easier figure reading. Further experimental conditions were as given in Figure 1.

coordinating the active-site copper (Boden et al., 1979; Bertini et al., 1981), inhibits the SOD activity; or to Arg-141 deprotonation; or to a quick alkaline denaturation of the modified SOD. The experimental results allow us to attribute such a fall in activity to the inhibition of OH^- anion coordinated with copper, although a partial denaturation effect cannot be excluded. The modified SOD, in fact, displayed a slow denaturation at pH values of approx. 12. Furthermore, from the best-fit values of the experimental curves in Figure 5, a mean pK_a value of about 11.5 is obtained. This, on the one hand, excludes the possibility that a decrease of k is due to Arg-141 deprotonation ($pK_a = 12.5$) and, on the other, it confirms the hypothesis of OH^- coordination according to the equilibrium:

the pK_a of which (11.3) is known from ^{17}O n.m.r. measurements (Bertini et al., 1981).

Kinetic behaviour of ACE-SOD

By reaction with acetic anhydride, an exhaustive modification of the superficial positively charged amino groups of SOD was performed, with their consequent transformation into neutral acetamidic groups (Cudd and Fridovich, 1982). Such modification entails an activity decrease of 90% with respect to the native enzyme, at pH values < 9 and $I = 0.04 \text{ M}$, as shown in Figure 6. In this figure ACE-SOD activity versus pH at three different I values is reported.

Such a drastic activity decrease can be ascribed to the disappearance of the electrostatic guidance. This hypothesis is supported by the observation that k is almost constant as I increases from 0.02 M to 0.25 M. The steadiness of k at various values of I is typical of reactions between two species, at least one of which is uncharged. Indeed, after elimination of the positive charges of lysine residues, almost no electrical charge should be experienced by O_2^- approaching the active site.

The pH effect on the activity of acetylated enzyme also appears to be different from that on N-SOD. Activity is almost constant at pH values < 10.5 and rapidly decreases at higher pH values. The kinetic rate constant value at pH values < 10.5 is about

Figure 7 Dependence of kinetic rate constant on ionic strength (I) of N-SOD and its derivatives at pH 8.1 and 12.3

Buffer solutions were tris(hydroxymethyl)aminomethane (0.039 M) and NaOH (0.02 M) respectively. Key to symbols: at pH 12.3, N-SOD ($\blacktriangle$); at pH 8.1, N-SOD ($\bullet$); PEG-SOD ($\triangle$); ACE-SOD ($\circ$); SUCC-SOD ($\square$).

$2.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, very close to the minimum k value of $2.9 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ measured at pH 12.3 for N-SOD. This indicates that the chemical modification of the superficial amino groups produces the same effect on N-SOD activity as does raising the pH to 12.3. In both cases, the elimination of the positive charges of protonated lysine amino groups close to the active site is obtained, and the same drastic fall in activity is recorded.

In Figure 6 a further activity decrease is evident at pH values > 10.5 . From the best-fit function of the experimental data a mean $\text{p}K_a$ value of about 11.5, as for SUCC-SOD, has been calculated.

In this context, the same qualitative arguments as put forward for SUCC-SOD about the inhibitor role of OH^- ion can be developed.

It is to be stressed that for all tested SODs, as I increases indefinitely the rate constant values appear to converge towards the limiting value of about $3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, which corresponds to the k value of N-SOD at pH 12.3. This is apparent from Figure 7 and from a more detailed trend concerning only N-SOD, which has been reported in a previous work (Argese et al., 1987).

In conclusion, the elimination of the O_2^- -driving charges of N-SOD carried out by deprotonation of the positively charged amino groups at high pH values, or by their chemical modification, or by their screening at high salt concentration, always entails a concomitant attenuation or disappearance of the electrostatic force driving O_2^- , with a consequent decrease in enzymic activity.

This strongly suggests that eventual conformation changes of

enzyme and active site are unimportant when compared with the effect of the charge variation on the activity.

In particular, the elimination of the electrostatic driving field, however obtained, lowers the enzymic activity to a value which is about 15 times smaller than the one measured in the presence of electrostatic control and represents the k value of the reaction limited by pure diffusion.

The kinetic results reported in the present work, besides providing a detailed survey on the kinetic features of SOD and its derivatives, can constitute a solid experimental basis to verify and improve the proposed models concerning the reactant-driving mechanism.

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