Carbamoylation of Cu,Zn-superoxide dismutase by cyanate

Role of lysines in the enzyme action

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Reaction with cyanate leads to a reversible change of the EPR spectrum of Cu,Zn-superoxide dismutase and to time-dependent carbamoylation of the lysine residues of the enzyme, producing a stable covalent derivative with more negative charge. The carbamoylated enzyme is less active than the native enzyme in spite of unaltered EPR spectra. The extent of this inactivation is much less when the enzyme activity is measured at low ionic strength. These results show that integrity of the active site is not the sole factor playing a role in the enzyme mechanism and that the ionic strength effect is related to electrostatic interactions between O_2^- and surface charges of the protein.

Superoxide dismutase *Carbamoylation* *Lysine neutralization*
Electrostatic interactions, in enzyme catalysis *Ionic strength, effect on enzymes*

1. INTRODUCTION

The copper of Cu,Zn-superoxide dismutase (SOD) is the active site of the enzyme. Singly charged anions of small size are inhibitors of the catalytic reaction, which involves the dismutation of O_2^-, a singly negatively charged molecule itself, to O_2 and H_2O_2 [1]. Therefore, anions can be considered substrate analogs for this enzyme and their reaction with Cu,Zn-SOD has been the subject intensive investigation. The current idea is that they bind to the copper by replacing a copper-coordinated water molecule and that this binding position is the same as for the substrate O_2^- [2]. This hypothesis is supported by a great deal of evidence in the case of CN^-, N_3^-, OH^- and halides [3]. In summary, they competitively inhibit the catalytic reaction and titrate the water proton relaxation rate of the enzyme copper as much as they do with other specific modifications of the copper chromophore, such as the EPR and visible spectra. This picture of the mode of anion binding by Cu,Zn-SOD has been challenged on the basis of spectroscopic data obtained with 'weak' anions like NCO^- and NCS^- [4–6], where measurements carried out under different experimental conditions were compared. Here, NCO^- has been reinvestigated comprehensively under controlled experimental conditions. The results confirm that NCO^- reacts with the superoxide dismutase copper in the same way as 'stronger' anions. A side-reaction with the lysine ϵ-amino groups occurred, with production of a stable carbamoylated derivative. The catalytic properties of this derivative show unexpected features interpreted as facilitating the catalytic reaction by a positively charged amino acid sidechain.

2. MATERIALS AND METHODS

Superoxide dismutase was isolated from bovine erythrocytes as in [7]. Enzyme activity was determined polarographically [8], in 0.1 M borate buffer (pH 9.8). X-band low temperature EPR spectra were recorded on a Varian E 9 spectrometer. Protein concentration was calculated colorimetrically [9]. Copper concentration was evaluated from A_{680}

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[7]. Polyacrylamide gel electrophoresis was done as in [10]. In samples to be analyzed for electrophoretic mobility and amino acid modification the reaction with cyanate was stopped by rapid passage through a Sephadex G-10 column. The extent of lysine modification by NCO^- was evaluated by amino acid analyses of duplicate protein samples: one analyzed after normal acid hydrolysis, under which conditions part of homocitrulline residues reconvert to lysines [11]; the other acid-hydrolysed after treatment with nitrous acid [12], which destroys the lysine residues not protected by carbamoylation of the ϵ-amino groups.

3. RESULTS

As described in [13], reaction of Cu,Zn-SOD with excess NCO^- resulted in nearly complete change of the EPR spectrum (fig. 1). This is a short-range coordination effect as NMR data have unequivocally shown [5] that NCO^- directly binds to the copper. The enzyme activity was also considerably inhibited (table 1). The inhibition was time-independent, confirming that it was due to the establishment of a single copper–ligand equilibrium. Enzyme activity in the presence of NCO^- was compared to that of the native enzyme in the presence of equimolar ClO_4^-. In fact, Cu,Zn-SOD is strongly inhibited by high ionic strength [14]; ClO_4^- inhibition is an ionic strength effect [14]. Dialysis of NCO^- led to full recovery of the EPR spectrum (fig. 1). Recovery of activity, however, displayed a singular pattern (table 1). It was nearly complete when assayed at high ionic strength; it was $\leqslant 50\%$ when assayed under routine conditions.

Fig. 2 shows polyacrylamide gel electrophoresis of the reaction. It is clear that incubation in the

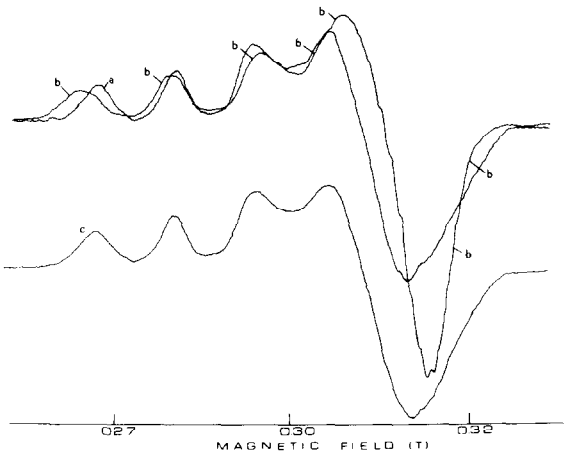

Fig. 1. EPR spectra of the reaction of the bovine Cu,Zn-SOD with NCO^-. The protein (4×10^{-4} M) in 0.1 M K-phosphate buffer (a), in the presence of 0.3 M KCNO (b) and after dialysis against the same buffer (c).

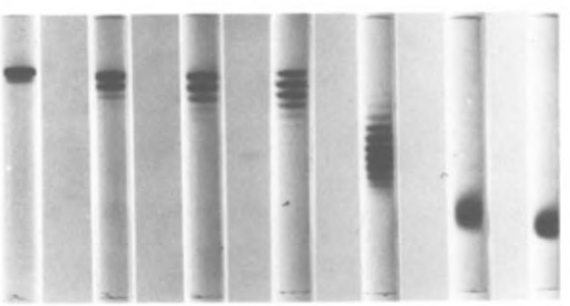

Fig. 2. Polyacrylamide gel electrophoresis of the reaction of bovine Cu,Zn-SOD with NCO^-. From the left: untreated SOD, and SOD incubated in the presence of 0.3 M NCO^- for 10 min, 30 min, 1 h, 8 h, 24 h and 48 h, respectively; 30 g protein was applied to each gel.

Table 1

Effect of NCO^- on superoxide dismutase activity of bovine Cu,Zn-SOD

Cyanate treatment	Catalytic constant ($k \times 10^{-9}\ M^{-1}.s^{-1}$)		
	0.1 M borate	0.1 M borate + 0.3 M ClO_4^-	0.1 M borate + 0.3 M NCO^-
–	1.58	0.68	0.24
Dialysis at 24 h	0.88	0.51	–
Dialysis at 48 h	0.73	0.46	–

presence of NCO^- gives rise to protein bands with increased anodic mobility until a single band is apparent at longer times. This mobility is fully maintained after removal of all NCO^- by dialysis. Amino acid analysis demonstrated that this loss of positive charge was due to the progressive carbamoylation of the ϵ-amino group of lysines, and that each band corresponded to the removal of a single positive charge. Substantially full carbamoylation was obtained after 24 h incubation with 0.3 M cyanate at 20°C.

4. DISCUSSION

These results show that NCO^-, an established anionic ligand for the copper of Cu,Zn-SOD, irreversibly carbamoylates the protein according to the reaction:

$$R\text{–}NH_3^+ + NCO^- \rightarrow R\text{–}NH\text{–}CO\text{–}NH_2$$

Since bovine Cu,Zn-SOD has a blocked N-terminal residue, the reaction with this enzyme involves the amino group of lysines. The fully carbamoylated protein has an EPR spectrum typical of the native enzyme (fig. 1c). This demonstrates that neutralization of positive charges of lysine side chains does not affect the integrity of the copper site. On the contrary, the activity of this derivative is 50% that of the native enzyme (table 1). This confirms that the copper is not the only factor in determining the catalytic properties of the enzyme, as suggested by results obtained with the succinylated protein [15] and with natural SODs with high isoelectric point [16]. In [15,16] it was apparent that electrostatic interaction of O_2^- with positively charged amino acid plays some role in facilitating the enzymatic reaction. This possibility was first suggested in [17] where neutralization of Arg_{141}, a positively charged sidechain near the copper site in the protein three-dimensional structure, led to enzyme inactivation. However, the copper optical spectrum was also affected and a purely electrostatic effect could not be demonstrated.

The above hypothesis is validated by the effect of ionic strength on the residual activity of the carbamoylated protein. This derivative is much less inhibited by ionic strength than the native protein (table 1). The result agrees with a role of the positively charged sidechain in facilitating the O_2^-–enzyme interaction. Neutralization of positive charges by either covalent reaction (NCO^-) or electrostatic interaction (ClO_4^-) brings the enzyme activity down to very similar lower limits, which tend, in our conditions, to ~50% of the activity in 0.1 M borate.

This conclusion poses an interesting evolutionary question which relates to the physiological role of superoxide dismutase. If lysine groups have such a role in superoxide dismutase activity, evolution should preserve the net protein charge as well as the residues crucial for the architecture of the active site. Since this is not the case [16], the simple reaction with O_2^- might not be the main evolutionary pressure for this class of proteins.

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