Phosphate Is an Inhibitor of Copper–Zinc Superoxide Dismutase†

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ABSTRACT: The superoxide dismutase (SOD) activity of bovine copper–zinc superoxide dismutase (Cu,Zn-SOD) in 50 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, was decreased by approximately 50% when the solution was made 10 mM in phosphate, in spite of the fact that the ionic strength of both solutions was adjusted to be equal. A similar experiment was carried out with bovine Cu,Zn-SOD chemically modified at Arg-141 with phenylglyoxal, which consequently had approximately 20% of the activity of the unmodified protein. (This activity was shown not to be due to residual unmodified protein.) Addition of 10 mM phosphate to solutions of the modified protein caused only a small decrease (<5%) in the SOD activity. The presence of phosphate also caused the affinity of Cu,Zn-SOD for binding azide or cyanide anions to be reduced; this effect of phosphate was also much less for the arginine-modified protein. We conclude that the inhibitory effect of phosphate on bovine Cu,Zn-SOD is due primarily to the neutralization of the positive charge on the side chain of Arg-141. The effect of increasing ionic strength on the activities of the native and arginine-modified proteins was also investigated. We found that at high concentrations of phosphate (≥10 mM), the SOD activities of native and arginine-modified Cu,Zn-SOD were inhibited comparably when the ionic strength was increased. This effect is presumably due to the lysine residues near the active site. At low concentrations of phosphate, by contrast, the native protein was much more sensitive to increasing ionic strength than was the arginine-modified protein. We conclude that Arg-141 is the primary site of interaction of phosphate in native bovine Cu,Zn-SOD and that, at low phosphate concentrations, Arg-141 is also the major source of the dependence of the SOD activity on ionic strength.

Copper–zinc superoxide dismutases (Cu,Zn-SOD) are a class of metalloproteins that are found to be very efficient catalysts of superoxide disproportionation

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

and it has been proposed that this activity is their primary function in vivo (Fridovich, 1979). The site of reactivity for superoxide, O_2^-, with Cu,Zn-SOD is the copper ion, which is also the site of binding of several anions including azide and cyanide (Valentine & Pantoliano, 1981). The isoelectric point for bovine Cu,Zn-SOD is 4.8 (Marmocchi et al., 1983). At neutral pH, the protein is negatively charged, and one might therefore predict that its reactivity with a negatively charged substrate such as superoxide would be enhanced by an increase in ionic strength. In fact, precisely the opposite is observed, and it is therefore believed that there are positively charged groups near the active site that play an important role in the interaction of the protein with superoxide and that are partially neutralized by interactions with anions at high ionic strength (McAdam, 1977; van Leeuwen, 1983).

X-ray crystallographic studies of bovine Cu,Zn-SOD indicate that the positively charged side chains of Arg-141, Lys-120, and Lys-134 are located in the vicinity of the active site, 5, 12, and 13 Å, respectively, away from the copper ion (Tainer et al., 1982). It has been proposed that these residues are responsible for electrostatic guidance of superoxide to the active site (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982). It has also been shown that the SOD activity and the affinity for anion binding (Bermingham-McDonogh et al., 1982; Cocco et al., 1983) are reduced by chemical modification of Arg-141 with phenylglyoxal (Malinowski & Fridovich, 1979) or of the lysines by acetylation, succinylation, or carbamoylation (Cudd & Fridovich, 1982; Marmocchi et al., 1982; Cocco et al., 1982). In the case of the arginine-modified protein, spectroscopic studies demonstrate that the environment of the copper ion in the oxidized protein is somewhat altered to give a more nearly axial geometry (Bermingham-McDonogh et al., 1982). By contrast, modifications of lysines by either succinylation or carbamoylation do not appear to alter the native rhombic geometry of the copper site (Cocco et al., 1983).

There have been several reports in the literature that indicate that the presence of phosphate anion can influence the observed properties of bovine Cu,Zn-SOD. Rotilio et al. (1972) noted that phosphate present at concentrations greater than 0.05 M interfered with cyanide binding to the protein. They also observed a substantially reduced SOD activity under the same conditions (Rigo et al., 1975). Calabrese et al. (1979) found that the nature of Co^{2+} binding to the apoprotein was different in the presence of phosphate than in its absence, and Strothkamp & Lippard (1981) observed that the imidazolate bridge linking the two coppers per subunit in the four-copper derivative (where copper has been substituted for zinc in the native protein) was broken in the presence of high concentrations of phosphate.

It has generally been assumed that the inhibitory effect of phosphate on SOD activity of bovine Cu,Zn-SOD is the result of the increase in ionic strength resulting from the addition of phosphate salts to the solutions (McAdam, 1977; Cudd & Fridovich, 1982). We have studied the effect of phosphate on the activity of bovine Cu,Zn-SOD and on its affinity for azide and cyanide anion binding. Our studies differ from those reported previously in that we carried out these studies under conditions of constant ionic strength. We found that increasing the phosphate concentration at constant ionic strength decreased the measured SOD activity of the protein as well as its affinity for anion binding. The SOD activity and anion binding properties of the protein that had been modified by phenylglyoxal reaction at Arg-141 were found to be much less affected by the presence of phosphate. We conclude, therefore, that Arg-141 is the site of phosphate interaction. We also found that, at low phosphate concentrations, the dependence of the SOD activity and anion binding affinity on ionic strength for the native and arginine-modified proteins was markedly

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different whereas, at high phosphate concentrations, they show similar dependencies. In the latter case, we believe the Arg-141 phosphate binding site may be saturated. The evidence supporting these conclusions is the subject of this paper.

Materials and Methods

Bovine liver Cu,Zn-SOD was purchased as a lyophilized powder from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, sodium azide, Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], xanthine, xanthine oxidase (grade I), and horse heart cytochrome *c* (type III) were supplied by Sigma; 6-hydroxydopamine hydrobromide was from Aldrich; potassium phosphate dibasic tetrahydrate and potassium cyanide were from Mallinckrodt, Inc.; sodium fluoride was from MBC Manufacturing Chemists, Inc. All were used as received.

Bovine Cu,Zn-SOD was chemically modified with phenylglyoxal and purified by the method of Malinowski & Fridovich (1979). The purified arginine-modified protein was dialyzed against water and lyophilized. Protein concentrations were determined by Lowry assay (Lowry et al., 1951), absorbance at 258 nm, or weight of lyophilized powder to be dissolved. SOD activity was assayed by observation of the inhibition of the autooxidation of 6-hydroxydopamine (Heikkila & Cabbat, 1976) or by the xanthine oxidase–cytochrome *c* assay system (McCord & Fridovich, 1969). These activity assays were modified in that 50 mM Hepes buffers at pH 7.4 were used instead of 50 mM phosphate buffers at pH 7.8 because phosphate was found to inhibit the SOD activity of Cu,Zn-SOD (vide infra).

Vis–UV spectra were recorded on a Beckman 5270 spectrophotometer with the sample compartment thermostated at 30 °C for the azide and cyanide binding studies. Absorbance measurements for activity assays were carried out at room temperature. ESR (electron spin resonance) spectra were recorded at 77K on a Varian E-12 instrument and calibrated with DPPH (α,α'-diphenyl-β-picrylhydrazyl) as a standard.

Results

The activity of bovine Cu,Zn-SOD and its anion binding properties were investigated in the presence of a number of different salts in an effort to discover an "innocent" ionic strength adjuster (see Table I). Two obvious choices, perchlorate and trifluoromethanesulfonate, were ruled out because of evidence that they were interacting with the protein directly. When the ionic strength was adjusted with perchlorate, for example, the SOD activity and the affinity for binding of azide and cyanide were partially suppressed relative to experiments where the ionic strength was adjusted with fluoride, suggesting a specific interaction of perchlorate with the protein. The phenomenon observed here with perchlorate is not uncommon. In fact, it has frequently been noted that perchlorate interferes more strongly than fluoride with enzyme activity and protein conformation (Record et al., 1978). SOD activities and anion binding affinities were higher when the ionic strength was adjusted with trifluoromethanesulfonate rather than with fluoride, but with the former, little or no effect of added phosphate on the properties of the protein was observed, suggesting that trifluoromethanesulfonate was interfering with the interaction of phosphate with the protein. For these reasons, we chose to study the effects of phosphate on the properties of the protein with sodium fluoride to adjust ionic strength. In spite of the fact that fluoride binds weakly to copper(II) in the protein (Rigo et al., 1977; Bertini et al., 1981; Viglino et al., 1981), fluoride seemed to maximize the effect of phosphate on the properties of the protein while giving the highest specific activity of those salts investigated (other than trifluoromethanesulfonate). As buffer for our studies, we chose Hepes because of its appropriate buffering range, its lack of affinity for metal ions, and its steric bulk, which made interference with anion binding sites on the protein unlikely.

Table I: Inhibitory Effect of Phosphate on the SOD Activity of Cu,Zn-SOD Determined by the Xanthine Oxidase–Cytochrome *c* Assay System[a]

concn of phosphate (mM)	required concn of SOD to give 50% inhibition (ng/mL)[b]			
	ionic strength of native SOD adjusted with			ionic strength of Arg-modified SOD adjusted with NaF
	NaF	$NaClO_4$	$NaCF_3SO_3$	
0	39	103	33	327
2.5	49	134		343
5.0	59	145		359
7.5	65	158		376
10.0	72	172	39	392

[a] All buffers were 50 mM in Hepes, pH 7.4, and were made 0.1 mM in ethylenediaminetetraacetic acid (EDTA) to prevent extraneous metal ion interference. All buffers were adjusted to an ionic strength of 0.15 by adding an appropriate amount of sodium salt. The pH of the buffered solutions was adjusted with a standardized NaOH solution, and the sodium ion contribution toward ionic strength was taken into account in calculating the amount of sodium salt required to bring ionic strength up to a value of 0.15. [b] The uncertainty in the SOD concentrations is estimated to be ±2%.

The effect of increasing concentrations of phosphate on the SOD activity of native bovine Cu,Zn-SOD and of the same protein chemically modified at Arg-141 by phenylglyoxal, determined by the xanthine oxidase–cytochrome *c* assay system (McCord & Fridovich, 1969) at constant ionic strength, is shown in Table I. The data indicate that phosphate at a concentration of 10 mM with $I = 0.15$, adjusted with fluoride, inhibits the SOD activity of the native protein by approximately 50% whereas the arginine-modified protein is only slightly inhibited under the same conditions. Similar results were obtained with the 6-hydroxydopamine assay (Heikkila & Cabbat, 1976), demonstrating that the effect observed was not due to the effect of phosphate on the assay system. In order to be certain that the SOD activity of the arginine-modified protein was in fact due to modified protein and not to native protein that had escaped modification, we subjected the purified modified protein to a second treatment with phenylglyoxal and observed no further loss of SOD activity. Similar results have also been reported by Cudd & Fridovich (1982), who also checked the residual activity of the arginine-modified enzyme by polyacrylamide gel electrophoresis followed by activity staining and found a single band of activity that had a greater anodic mobility than the native enzyme, confirming that the activity was not due to unmodified protein.

The specific activities of the native and modified proteins were measured under the standard conditions of the xanthine oxidase–cytochrome *c* assay (McCord & Fridovich, 1969), i.e., 50 mM phosphate, pH 7.8, and ionic strength equal to approximately 0.12, and were found to be 3500 and 700, respectively. The specific activities of these proteins at $I = 0.15$ in Hepes at pH 7.4, calculated from the data in Table I, are 8200 and 1000, respectively. Thus, it is clear that the high levels of phosphate used in the standard assay are masking almost half of the inherent specific activity of the protein.

The azide and cyanide affinities of the native and arginine-modified proteins were studied in the presence and absence of phosphate in the ionic strength range 0.15–0.50 (see

Table II: Association Constants for Azide and Cyanide Binding to Bovine Liver Cu,Zn-SOD[a]

buffer[b]	I[c]	K_{azide} (M^{-1}) native SOD	K_{azide} (M^{-1}) Arg-modified SOD	$K_{cyanide}$ (M^{-1}) native	$K_{cyanide}$ (M^{-1}) Arg-modified SOD
50 mM Hepes	0.15	87.2	24.1	1.8×10^5	0.49×10^5
	0.30	75.9	26.3		
100 mM Hepes	0.30	70.0	28.3	1.5×10^5	0.36×10^5
	0.50	68.6	30.6	1.0×10^5	0.31×10^5
50 mM P_i	0.15	63.0	23.6	0.78×10^5	0.34×10^5
	0.30	50.9	23.7		
100 mM P_i	0.30	47.3	26.8	0.52×10^5	0.26×10^5
	0.50	47.3	27.3	0.41×10^5	0.21×10^5

[a] The azide affinity constant was calculated from the absorbance of the charge-transfer band at 373 nm (Foster, 1969), and the cyanide affinity constant was calculated from the absorbance of the bands at 660 and 530 nm by the method described in Rotilio et al. (1972). [b] All buffers were at pH 7.4 and were made 0.1 mM in EDTA to prevent extraneous metal ion interference. [c] Ionic strength adjusted with NaF.

Table II). The data indicate that the azide and cyanide binding affinities of the native protein decrease markedly with increasing ionic strength, whereas the affinities of the arginine-modified protein are nearly independent of ionic strength. In addition, for any given ionic strength studied, the addition of phosphate to the native protein markedly reduced its affinity for anion binding whereas the addition of phosphate to the arginine-modified protein had little effect on its anion binding affinity.

Sulfate was also briefly investigated as a possible analogue of phosphate. It was found that sulfate also reduced the azide affinity of the native protein, but to a smaller degree than phosphate.

Discussion

Although it was previously observed that the presence of phosphate caused a lowering of the SOD activity of bovine Cu,Zn-SOD (Rigo et al., 1975) and that it interfered with cyanide binding (Rotilio et al., 1972), these effects were attributed to a general effect of ionic strength and not to a specific effect of phosphate (McAdam, 1977; Cudd & Fridovich, 1982). Our studies differ from those carried out previously in that they were carried out under conditions of constant ionic strength. We found that there remained a distinct inhibitory effect of phosphate apart from the effect of ionic strength.

The specific activity vs. phosphate concentration for native and arginine-modified proteins at two different ionic strengths is plotted in Figure 1. This plot shows clearly that the specific activity of the native proteins is dependent on phosphate concentration whereas the specific activity of the arginine-modified proteins is essentially insensitive to phosphate. This evidence suggests that (a) Arg-141 is the binding site for phosphate and (b) once phosphate is bound to Arg-141, the positive charge on the side chain of Arg-141 is neutralized and this amino acid residue can no longer participate in electrostatic guidance of the substrate superoxide anion to the copper(II) site. The small dependence of the residual SOD activity on phosphate concentration for the modified protein is probably due to interaction of phosphate with Lys-120 and Lys-134, which also have been proposed to be involved in electrostatic guidance of superoxide anion to the copper site of the native protein (Cudd & Fridovich, 1982; Marmocchi et al., 1982; Cocco et al., 1983).

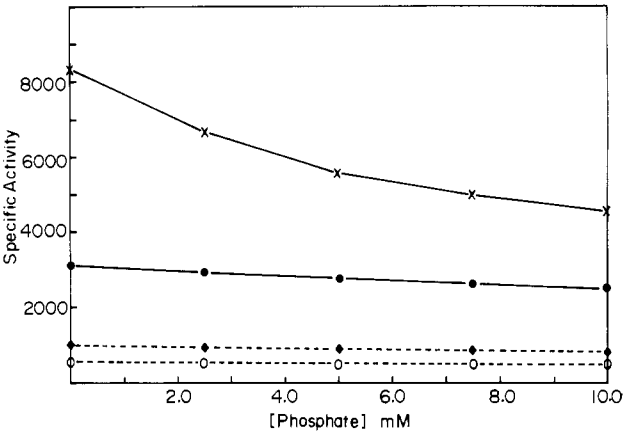

FIGURE 1: Dependence of specific activity (in units of SOD activity per milligram of protein) on phosphate concentration for native bovine SOD (solid lines) at ionic strength $I = 0.15$ (×) and $I = 0.30$ (●) and for Arg-modified SOD (broken lines) at $I = 0.15$ (♦) and $I = 0.30$ (○). SOD activity was measured by the xanthine oxidase–cytochrome *c* assay system in 50 mM Hepes (pH 7.4), and phosphate was added in the form of potassium phosphate dibasic trihydrate. All buffers were made 0.1 mM in EDTA to prevent extraneous metal ion interference, and ionic strength was adjusted with NaF. Specific activity was calculated from the data in Table I.

The specific activity of both native and modified protein decreased at higher ionic strength (see Figure 1). When the ionic strength was doubled to 0.30, the specific activity of both native and arginine-modified SOD decreased by approximately a factor of 2 at high phosphate concentration. An increase of the negative charge of the protein achieved through phosphate binding to lysine residues in both native and arginine-modified proteins may explain the same ionic strength behavior at high phosphate concentration. However, when phosphate concentrations are low, increased ionic strength has a far greater effect on the activity of the native protein than on the activity of the arginine-modified protein. We conclude that at low phosphate concentrations most of the inhibitory effect comes from the interaction of anions with Arg-141. The involvement of Arg-141 in the dependence of SOD activity on ionic strength was not observed by Cudd & Fridovich (1982), presumably because their studies were carried out at higher phosphate concentrations (>20 mM).

In addition to our studies of the SOD activities of the native and modified proteins, we also determined their anion binding affinities for azide and cyanide in the presence of phosphate (see Figure 2). We found that at the same ionic strength phosphate has much less effect on the anion affinity constants for the modified protein relative to the native protein. This evidence suggests once again that Arg-141 is the site for phosphate binding in bovine Cu,Zn-SOD. The data in Figure 2 also indicate that the anion affinity of the native protein decreased with increasing ionic strength whereas the anion affinity of the modified protein is almost independent of ionic strength. This behavior is similar to the dependence of specific activity on ionic strength discussed above and shown in Figure 1.

An interesting observation was made during the investigation of the azide affinity of native and arginine-modified proteins, namely, that the azide-to-copper charge-transfer band for the modified protein occurs at 355 nm as opposed to 373 nm for the native protein. This band has been previously assigned as an azide-to-copper charge-transfer band characteristic of azide bound to an equatorial position in the copper coordination sphere (Morpurgo et al., 1973; Solomon, 1981). It seems possible from the crystal structure that the azide bound to the copper(II) ion of the native protein may be directly

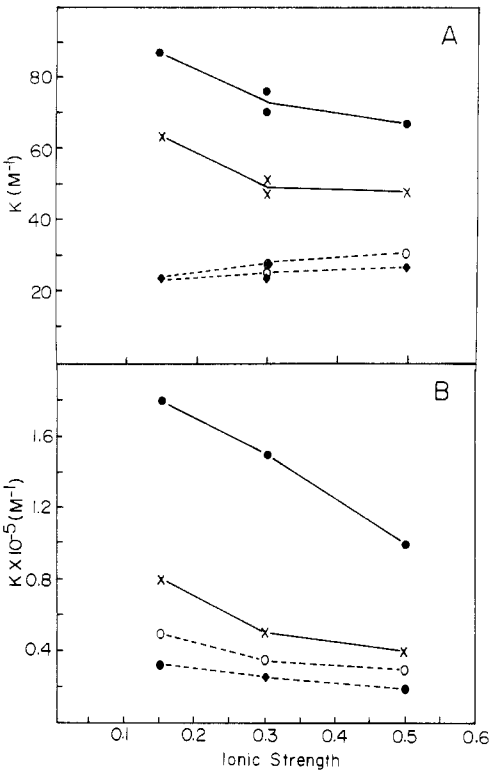

FIGURE 2: (A) Dependence of azide affinity constants on ionic strength for native bovine SOD (solid lines) in Hepes (●) and phosphate (×) and for Arg-modified SOD (broken lines) in Hepes (○) and phosphate (♦). All buffers had their ionic strength adjusted with NaF and were at pH 7.4. (B) Dependence of cyanide affinity constants on ionic strength for native and Arg-modified SOD. Symbols in (B) refer to the same buffer conditions as in (A).

interacting with Arg-141. This mode of hydrogen bonding is presumably not available for the modified protein, and its absence may be the cause of the spectral difference.

An alternative explanation for the inhibition by phosphate of SOD activity and anion binding is that phosphate may be binding directly to the copper(II) ion in the native protein. We consider this possibility to be unlikely because no visible or ESR spectral changes were found in solutions of Cu,Zn-SOD at high concentrations of phosphate. We thus conclude that the inhibitory effect of phosphate on bovine Cu,Zn-SOD is due primarily to neutralization of the positive charge on the side chain of Arg-141, reducing the effect of this amino acid residue in providing electrostatic guidance of substrate and anions to the active site of the enzyme. The small inhibition of SOD activity by phosphate in the case of the arginine-modified protein indicates that lysines play little or no role in the mechanism of phosphate inhibition. It is important to note that the affinity of arginine and lysine residues for phosphate in Cu,Zn-SOD is not at all unusual as there are well-documented cases of phosphate binding to other proteins through the same amino acid residues (Riordan, 1979).

It is intriguing to speculate about the possibilities for a functional significance for the effect of phosphate on the properties of this protein, but we have no compelling theories at this time. Estimates of the concentration of free intracellular inorganic phosphate determined by nuclear magnetic resonance (NMR) are in the range 1–1.5 mM (Gadian, 1982), a concentration range where we can clearly see effects of phosphate. Moreover, we also see effects from the presence of other biological phosphates that are present in the cell (D. Mota de Freitas and J. S. Valentine, unpublished data). It is apparent that previous results of studies of SOD in concentrated phosphate buffers should be reevaluated to determine if the presence of phosphate may have influenced the results. It is also interesting to note that the manganese and iron SODs are similarly inhibited by anions (including perchlorate). However, the source of this inhibition is not yet known (Benovic et al., 1983).

Registry No. Cu,Zn-SOD, 9054-89-1; Arg, 74-79-3; phosphate, 14265-44-2; azide, 14343-69-2; cyanide, 57-12-5.

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