The Characterization of Cobalt Ions in Cobalt-substituted Superoxide Dismutase (Co_2Zn_2SOD)

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Bovine erythrocyte superoxide dismutase (Cu_2Zn_2SOD) is the metalloenzyme which contains copper and zinc ions in each subunit. Rotilio *et al.* [1–3] prepared the cobalt enzyme (Co_2Zn_2SOD) in which only the copper ions in the native enzyme were substituted by cobalt ions. Co_2Zn_2SOD has a characteristic visible spectrum in an aqueous solution (pH 7.4) and its spectrum dramatically changes to another spectrum with addition of phosphate ions at pH 7.4 [1–3]. These characteristic spectral changes upon addition of phosphate ions are interpreted by the changes of the coordination geometry of the cobalt ions from tetrahedral to five-coordination, on the basis of characteristic bands of five-coordinate geometry in the near infrared region [1].

In a previous paper [4], we showed that magnetic circular dichroic (MCD) spectra can determine the five-coordination geometry of cobalt-substituted bovine carbonic anhydrase. In this paper, we measured the MCD spectra to confirm the coordination geometry of the cobalt ion in Co_2Zn_2SOD and the binding constant of cobalt ions for the native copper sites in the enzyme.

Bovine erythrocytes superoxide dismutase, Cu_2Zn_2SOD, was purified from bovine erythrocytes by the method of McCord and Fridvich [5]. Cobalt-substituted superoxide dismutase, (Co_2Zn_2SOD), was prepared by the method of Calabrese *et al.* [2] using 0.01 M HEPES buffer (pH 7.4) instead of phosphate buffer. The MCD spectra were recorded on a JASCO 40C spectropolarimeter in a magnetic field of 11.4 kG at room temperature. The relaxation time of a phosphate ion was measured on a JEOL-FX-100 NMR spectrometer. The equilibrium dialysis was carried out with an equilibrium dialysis cell (1 ml capacity) at 4 °C. The detailed methods of the

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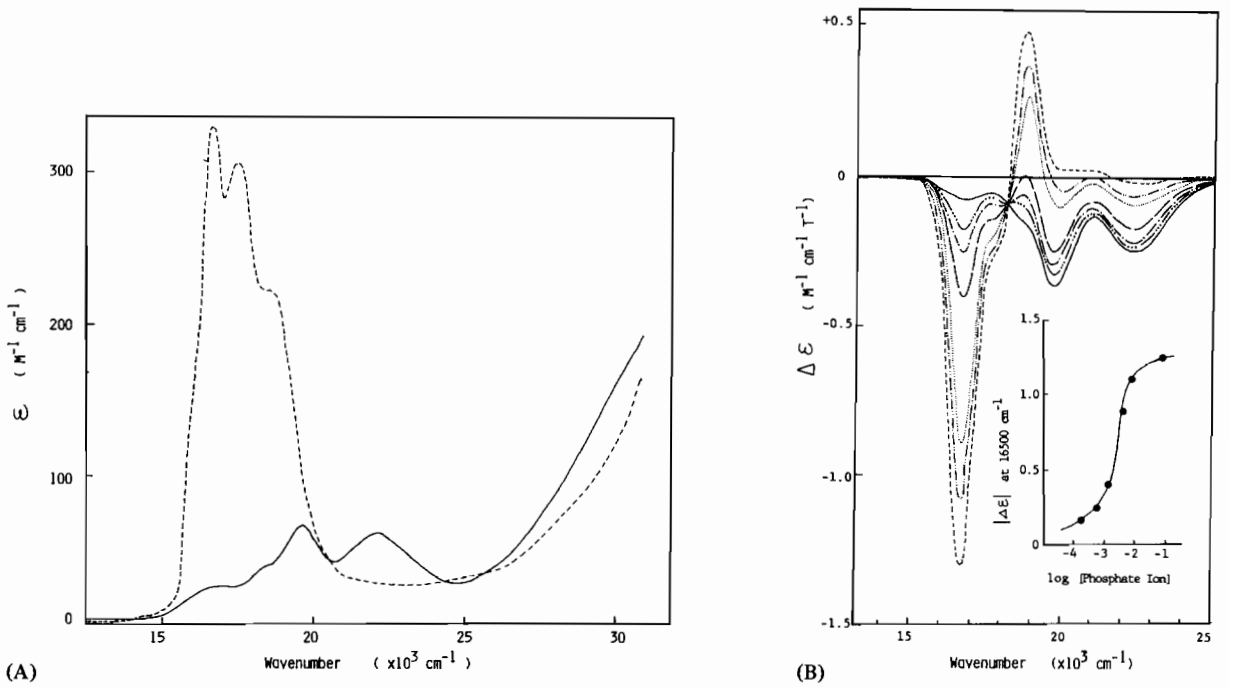

Fig. 1. Electronic and magnetic circular dichroic (MCD) spectra of Co_2Zn_2SOD. (A): Electronic spectra of phosphate and water form of Co_2Zn_2SOD at pH 7.4. -------, 8.4×10^{-2} M phosphate ion in 0.01 M HEPES buffer; ———, 0.01 M HEPES buffer (pH 7.4). (B): Magnetic circular dichroic spectra of Co_2Zn_2SOD with increasing of phosphate ions. Phosphate ions: ———, 0 M; –··–, 2.0×10^{-4} M; –·–, 5.3×10^{-4} M; – – –, 1.52×10^{-3} M; ·····, 3.52×10^{-3} M; –···–, 7.52×10^{-3} M; ------, 8.40×10^{-2} M.

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TABLE I. MCD Band Energy for Water and Phosphate Form of Cobalt Substituted Bovine Superoxide Dismutase (Co_2Zn_2SOD) and Ternary Complex between Cobalt(II)–Bovine Carbonic Anhydrase (Co(II)–BCA) and Various Ligands.

	Band Position ($cm^{-1} \times 10^{-3}$) and $\Delta\epsilon$ (M^{-1} T^{-1} cm^{-1})				
Phosphate form of Co_2Zn_2SOD	16.5(−1.3)		18.8 (+0.47)		
Water form of Co_2Zn_2SOD	16.5(−0.07)		18.8(−0.14)sh	19.6(−0.35)	20.2(−0.25)
CN^- adduct of Co(II)–BCA(Td)[a]	15.8(−0.84)		18.4 (+0.38)		
CH_3COO^- adduct of Co(II)–BCA(F)[a]		17.3(−0.30)		19.2(−0.13)sh	20.5(−0.11)
8-Quinolinecarboxylate adduct of Co(II)–BCA(F)[a]		17.5(−0.20)		19.5(−0.15)sh	21.8(−0.19)
Oxalate adduct of Co(II)–BCA(F)[a]		17.5(−0.40)	18.6(−0.21)sh		22.1(−0.14)

sh, shoulder; Td, tetrahedral; F, five-coordinate. [a]Ref. [4].

equilibrium dialysis are described in previous papers [6, 7].

Figure 1A shows the spectra of Co_2Zn_2SOD in 0.01 M HEPES buffer (pH 7.4) in the absence (water form) and presence (phosphate form) of phosphate ions. The spectrum of the water form dramatically changes to that of the phosphate form with the addition of phosphate ions. This behavior is consistent with that obtained by Desideri *et al.* [1]. The MCD spectra of Co_2Zn_2SOD in various concentrations of phosphate ions at pH 7.4 are shown in Fig. 1B. In the absence of phosphate ions the MCD spectrum (water form) has negative bands at 16500, 18500 (shoulder), 19800, and 22300 cm^{-1}. With increasing concentrations of phosphate ions, negative intensities of the bands at 19800, 18500 (shoulder), and 22300 cm^{-1} decreased. On the other hand the negative band at 16500 cm^{-1} increased; a new positive band appeared at 19000 cm^{-1} whose intensity was proportional to the phosphate ion concentration. Finally, the MCD spectrum (phosphate form) in the higher concentration of phosphate ions had the large negative and small positive bands at 16500 and 19000 cm^{-1}, respectively. This MCD spectral shape is very similar to that which was assigned as tetrahedral geometry in model cobalt complexes [8]. It also resembles the MCD spectrum (Table I) of the CN^- adducts of cobalt(II)–bovine carbonic anhydrase which has typically tetrahedral geometry [4]. These results indicate that the cobalt ions in the phosphate form of Co_2Zn_2SOD have tetrahedral geometry.

The MCD bands at 18500, 19800, and 22300 cm^{-1} in the water form completely disappear in the phosphate form. The intensities and band positions of characteristic MCD bands in the water form are very similar to those of the adducts (CH_3COO^-, 8-quinolinecarboxylate) of Co(II)–bovine carbonic anhydrase, which have five coordination geometry (Table I). Two distinct negative bands were also observed in the MCD spectra of Co(II)-substituted nitrite reductase, which have five-coordinate geometry [9], and of the Co(II) model complexes [8]. Therefore, the main species of the water form have five-coordinate geometry. The bands at 18500, 19800 and 22300 cm^{-1} arise from five-coordination geometry of the cobalt ion of water form of Co_2Zn_2-SOD, but the small negative band at 16500 cm^{-1} in the water form could not be interpreted by five-coordination geometry of cobalt ions in the enzyme. This band was not observed in the MCD spectra of various adducts (Table I) of cobalt(II)–bovine carbonic anhydrase which have five-coordinate gometry [4]. The phosphate form of CO(II)–SOD has a large negative MCD band at the same wavenumber, so that there is a possibility that small amounts of the tetrahedral species may coexist in the water form.

If the MCD band at 16500 cm^{-1} ndicates the existence of the tetrahedral species, the content of the tetrahedral species in the water form can be calculated as being about 5% from the intensity of the MCD band at 16500 cm^{-1} of the phosphate form. Therefore, the main species in the water form will have five-coordinate geometry.

The cobalt ions which bind to a copper site in Co_2Zn_2SOD were released from their sites by prolonged dialysis in a large amount of 0.01 M HEPES buffer (pH 7.4). This behavior indicates that the binding constant of cobalt ions to the copper site is not large enough in 0.01 M HEPES buffer (pH 7.4). Therefore, the binding constant was determined by the equilibrium dialysis method, using 2-pyridinecarboxylate as a competing chelating agent for cobalt ions. The equilibrium dialysis was carried out for 30 days in a cold room. The equilibrium state was reached after 20 days dialysis. The cobalt and zinc contents were measured at various periods. The cobalt content which bound to the enzyme in the equilibrium state decreased with increasing concentration of 2-pyridinecarboxylate, but the contents of the zinc-bound (over 90% zinc ions bind to the enzyme) were not changed even at high concentration of 2-pyridinecarboxylate (Fig. 2). The relationship between the logarithm of free ligand concentration and r (number of cobalt ions (zinc ions) bound to 1 mol enzyme) is shown in Fig.

TABLE II. Stepwise Apparent Binding Constants of Metal Ions of Bovine Superoxide Dismutase.

Enzyme	Binding Metal Ions	$\log K_1$	$\log K_2$
E_2Zn_2SOD (pH 7.4)	Co^{+2}	6.7	5.2
E_2Zn_2SOD (pH 6.25)[a]	Zn^{+2}	7.8	6.5

[a]Ref. [6].

TABLE III. T_1^{-1} Values for ^{31}P and Water Protons of Phosphate Buffer in the Presence of Metal Substituted Bovine Superoxide Dismutase.

	Conditions	T_1^{-1} (sec^{-1})
^1H	Co_2Zn_2SOD 4.5×10^{-4} M (pH 7.4, 0.05 M phosphate buffer)	0.33
	Co_2Zn_2SOD 4.5×10^{-4} M (pH 7.4, water)	0.35
	phosphate buffer (pH 7.4, 0.05 M)	0.29
^{31}P	Co_2Zn_2SOD 3.7×10^{-4} M (pH 7.4, 0.1 M phosphate buffer)	15.6
	E_2Zn_2SOD 3.8×10^{-4} M (pH 7.4, 0.1 M phosphate buffer)	0.18

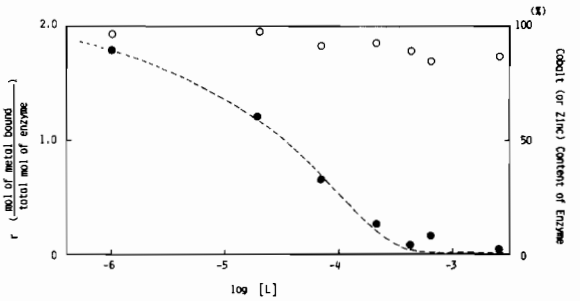

Fig. 2. The relationship between metal content in the enzyme and free ligand concentration at pH 7.4 (0.01 M HEPES buffer). Co_2Zn_2SOD (1.38×10^{-4} M) was dialyzed with various concentrations of 2-pyridinecarboxylate (1.0×10^{-4}–2.5×10^{-3} M) in equilibrium dialysis cells. Free ligand concentrations were calculated from the metal contents in the ligand chamber of equilibrium dialysis cell and the total ligand concentration by a microcomputer NEC PC-9801 F. •, cobalt content; ○, zinc content.

2. The binding constants of cobalt ions for copper sites in Co_2Zn_2SOD are shown in Table II: they are almost 10 times smaller than the binding constants of zinc ions for the copper sites, implying that small amounts of cobalt ions (5%) dissociate from Co_2Zn_2SOD (2×10^{-1} M). Therefore, prolonged dialysis against large amounts of buffer results in the release of cobalt ions from the cobalt-enzyme.

The spin–lattice relaxation times (T_1) of phosphate ions were measured to determine whether or not phosphate ions directly bind to the cobalt ions in phosphate form of Co_2Zn_2SOD, and are shown in Table III. The spin–lattice relaxation times of water proton are also shown in Table III. T_1^{-1} of ^{31}P of a phosphate buffer in the presence of Co_2Zn_2SOD was much larger (about 90 times) than that obtained for cobalt-free enzyme (E_2Zn_2-SOD). In other analogous cases [10, 11], a larger T_1^{-1} of ^{31}P was measured. The large change of T_1^{-1} of ^{31}P in the presence of Co_2Zn_2SOD indicates that if the coupling between the nucleus and the unpaired electrons is dipolar in origin, phosphate ions directly bind to cobalt ion in the copper site of enzyme, because the spin–lattice relaxation time is largely influenced by the distance between cobalt ion and phosphate atom. Therefore, there is a possibility that phosphate ions directly bind to the cobalt ion in the active site. However, it is necessary to obtain more data to determine whether phosphate ions directly bind to the cobalt ion in the enzyme. The water ^1H T_1^{-1} values of solutions containing the paramagnetic derivative (Table III) do not show any enhancement with respect to the diamagnetic value. This may be because the phosphate form has no water in the coordination sphere and the water form probably does have water, but τ_c is so short (10^{-12} s in five coordinate species [12, 13]) that T_1^{-1} of the proton is only slightly affected [14, 15].

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