Investigation of Zinc-deprived Bovine Superoxide Dismutase

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Zinc-deprived bovine superoxide dismutase and its adducts with azide and thiocyanate ions have been investigated through water ^1H NMR relaxation measurements. The affinity constants of the anions for the modified protein have been determined and compared with those for the native enzyme. The results suggest that a histidine different from the bridging one is displaced upon anion coordination.

Introduction

The study of zinc-deprived bovine superoxide dismutase is useful both in understanding an enzymatically active protein [1] and as a further step in the knowledge of the native enzyme. The zinc-deprived protein is known to display an axial EPR spectrum with $g_{\parallel} = 2.26$ and $g_{\perp} = 2.06$ [2] whereas the native enzyme is strongly rhombically distorted [3].

The d–d absorption spectrum of copper(II) is essentially unchanged [4]. Presumably the release of the steric constraint due to the histidinato bridge between zinc and copper in the native enzyme provides a more 'symmetrical' copper(II) coordination polyhedron.

We have investigated through water ^1H NMR relaxation measurements in the field range 4–60 MHz the zinc-deprived enzyme and its NCS^- derivative. The affinity constants of NCS^- and N_3^- have also been measured and their EPR spectra recorded.

Experimental

Bovine erythrocytes superoxide dismutase (Cu_2Zn_2SOD) was obtained from Sigma Chemical Company, Miles Laboratories (PTY) Limited, and DDI Diagnostic Data, Inc. as lyophilized products. The last was used as such and the zinc free derivative (Cu_2E_2SOD) was obtained by dialysis against phosphate buffer at pH = 3.6 [5, 6]; the others were purified by chromatography on DEAE cellulose [7], equilibrated with phosphate buffer at pH = 7.4 and were freed of both metals [7] by dialysis against several changes of a solution of 0.05 *M* phosphate acetate buffer, 0.10 *M* EDTA at pH = 3.8, followed by dialysis against a solution of 0.05 *M* phosphate acetate buffer and 0.10 *M* NaCl at pH = 3.0 to remove protein-bound EDTA [6]. Finally, dialysis against the same buffer but in the absence of NaCl was performed in order to remove the salt [6]. The apoprotein was then diluted with the last buffer to give a final concentration of 1 mg/ml and slowly titrated with a 3.0 m*M* solution of $CuSO_4$ [8].

Cu_2E_2SOD was then dialyzed against 0.25 *M* acetate buffer at pH = 5.5 or against doubly distilled water. Both procedures gave the same results as shown from electronic spectra and EPR spectra at 77 K, and all subsequent measurements were performed on unbuffered samples.

Ninety per cent ^{13}C-enriched potassium thiocyanate was purchased from Prochem B.O.C.; all the other chemicals were analytical grade, and freshly bidistilled water was used throughout.

Spectroscopic, NMR and ESR measurements were performed by adding aliquots of concentrated anion solution to enzyme solutions by means of an automatic micropipette. Final dilutions were not larger than 20% and were considered in the calculations.

The electronic spectra were recorded on a Cary 17D spectrophotometer in the absorbance range 10,000–25,000 cm^{-1} with 10 mm pathlength cells Protein concentration was determined at 258 nm (ϵ = 13200 M^{-1} cm^{-1} [7] and 680 nm (ϵ = 300 M^{-1} cm^{-1} [7] for Cu_2Zn_2SOD and ϵ = 5800 M^{-1} cm^{-1} [2] for apo SOD). The concentrations of Cu_2E_2SOD samples were determined from the absorption at 680 nm using ϵ = 280 M^{-1} cm^{-1}. X-band ESR spectra were recorded on a Bruker 200 TT spectro-

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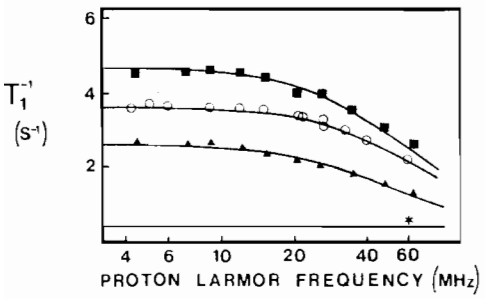

Fig. 1. Water proton longitudinal relaxation rates as a function of proton Larmor frequency for 8.2 × 10^{-4} *M* monomeric SOD solutions at pH 6.0 and 26 °C: ○ = Cu_2-Zn_2SOD; ■ = Cu_2E_2SOD; ▲ = Cu_2E_2SOD + 0.36 *M* NCS^-. The values for the reduced native protein (——) and for $Cu_2E_2SOD + N_3^-$ (*) are also shown.

meter. The NMR measurements in the proton Larmor frequency range 4–60 MHz were performed on a Bruker CXP 100 spectrometer equipped with a 1.4 Varian DA 60 magnet [9]. The 80 MHz ^1H and 20 MHz ^{13}C NMR spectra were recorded on a Varian CFT 20 spectrometer. All the measurements were run at room temperature unless otherwise specified.

Longitudinal relaxation times, T_1, were measured with the inversion recovery method using an appropriate nonlinear least-squares fitting program. Transverse relaxation times, T_2, were obtained from the linewidth at half-peak height, through the relation $T_2^{-1} = \pi\Delta\nu$.

Results and Discussion

The water ^1H NMR data of a solution containing zinc deprived SOD are reported in Fig. 1 and compared with the data for the native enzyme. The latter are in agreement with those previously reported [10]. A qualitative analysis of the data indicates that water is present in the coordination sphere of the zinc-deprived enzyme in a similar way to the native zinc enzyme. In both patterns the ^1H T_1^{-1} data are strongly field dependent, indicating that the field range investigated corresponds to a dispersion range in the Solomon equation [11] which is likely to be due to the ω_1 containing term. Although the T_1^{-1} data on the native enzyme extended to very low field have shown that the behaviour predicted by the Solomon equation is not followed [12], the dispersion in the high field range can still be fitted through an equation of the type

$$T_1^{-1} = \frac{[E]}{111}\gamma_I^2 g^2 \beta^2 S(S+1)\left(\sum_i \frac{1}{r_i^6}\right)\frac{\tau_c}{1+\omega_I^2\tau_c^2} \qquad (2)$$

where [E] is the enzyme concentration, 111 is the molarity of water protons, and the other symbols have their usual meaning. Such an equation can be expressed in terms of a geometrical factor $G = \Sigma_i 1/r_i^6$ and of a function of τ_c: $T_1^{-1} = K \cdot G \cdot f(\tau_c)$ [9]. An analysis of the data provides $G = 2.6 \pm 0.3 \times 10^{-15}$ pm^{-6} and $\tau_c = 3.7 \pm 0.4 \times 10^{-9}$ s which compares with $G = 2.2 \pm 0.3 \times 10^{-15}$ pm^{-6} and $\tau_c = 2.6 \pm 0.3 \times 10^{-9}$ s for the native enzyme. Despite the simplicity of the approach it can be safely concluded that τ_c for both systems is similar and can be set around 10^{-9} s as usually found for copper(II) chromophores [13]. In both cases the absolute values of the T_1^{-1} and G values are indicative of a semi-coordinated water. If the coordination number of copper(II) in the two systems is the same then the disappearance of large rhombic components is in our opinion due to a rearrangement of the ligands towards a square pyramid. Indeed, theoretical analysis of the g values showed that large rhombic components in the $g_\perp$ region are due to distorted trigonal bipyramidal geometries [14].

NCS^- and N_3^- are capable of binding copper(II) as in the native SOD [15, 16]. ^{13}C NMR data on NCS^- interacting with the zinc-deprived protein show considerable line broadening due to nuclear unpaired

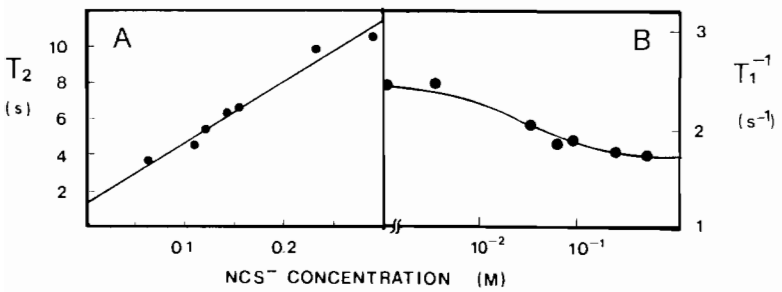

Fig. 2. A: 20 MHz transverse relaxation time T_2 of the ^{13}C nucleus of thiocyanate in the presence of 6.66 × 10^{-5} *M* monomeric zinc-deprived SOD at pH 6.0 and 26 °C as a function of NCS^- concentration. B: 80 MHz longitudinal relaxation rate of water protons in 1.74 × 10^{-3} *M* monomeric zinc-deprived SOD at pH 6.0 and 26 °C as a function of NCS^- concentration (logarithmic scale).

electron coupling. The T_2 values are linearly dependent on concentration (Fig. 2A), and were used to estimate the binding constant as 26 ± 3 M^{-1} at pH 6. Thiocyanate also reduces the water ^1H T_1^{-1} values in a pattern which permits the calculation of the affinity constant as 33 ± 3 M^{-1} (Fig. 2B), in fair agreement with the above value. The frequency dependence of the ^1H T_1^{-1} values of a solution containing the zinc-deprived protein and excess NCS^- (0.36 M) is reported in Fig. 1. The analysis provides a G value of 1.1 ± 0.2 × 10^{-15} pm^{-6} and τ_c of 3.5 ± 0.4 × 10^{-9} s. The τ_c value indicates that water is firmly bound to the chromophore since the correlation time is still determined, as in the pure system, by the electronic relaxation time. The G value on the other hand indicates that water is rather distantly bound compared with the non ligated chromophore. N_3^- also binds copper(II); from the development of a charge transfer band at 375 nm an affinity constant of 234 ± 7 M^{-1} at pH 6.0 is estimated. N_3^- reduces the ^1H T_1^{-1} values to a value close to the diamagnetic contribution of the reduced protein (Fig. 1). The affinity constants of both ligands are of the same order of magnitude as those found for the native protein at the same pH, which have been determined to be 20 ± 3 M^{-1} for NCS^- (through ^{13}C linewidth) and 90 ± 6 M^{-1} for N_3^- from UV absorption spectroscopy. The EPR spectra of both ligand derivatives are axial with the following values: NCS^-, $g_\parallel$ = 2.26, $g_\perp$ = 2.05, $A_\parallel$ = 155 × 10^{-4} cm^{-1}; N_3^-, $g_\parallel$ = 2.25, $g_\perp$ = 2.05, $A_\parallel$ = 162 × 10^{-4} cm^{-1}. The latter values are similar to those already reported by Beem *et al.* [17]. The present data do not definitely settle the problem of whether inhibitors bind at a histidine or the water site. However, the present data could be consistent with NCS^- being present with a water molecule in the coordination sphere, thus substituting a histidine nitrogen as it does with native SOD [15, 16]. If the ligands remove a histidine, possibly causing the simultaneous displacement of the axial water, then the bridging histidine would not be substituted by the ligand since the anion binding affinity for native and zinc deprived enzymes is similar.

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