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^{19}F RELAXATION AS A PROBE OF THE OXIDATION STATE OF Cu, Zn SUPEROXIDE DISMUTASE. STUDIES OF THE ENZYME IN STEADY-STATE TURNOVER.

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Summary. ^{19}F nmr relaxation proved to be a proper method to evaluate the Cu^{2+}/Cu^{+} ratio at the active site of Cu, Zn superoxide dismutase in either equilibrium or turnover conditions. In the steady-state under fluxes of O_2^-, the enzyme was found to contain 50% Cu^{2+}, in accord with the equal rates of copper catalytic reduction and oxidation. Previous results giving 75% Cu at the steady-state without change of the overall catalytic constant were confirmed for samples subjected to freeze-drying or freezing-thawing.

The catalytic mechanism of Cu, Zn superoxide dismutase (SOD) has been shown by pulse radiolysis to involve alternate reduction and oxidation of the copper ion by O_2^- with equal rates (1, 2). Therefore, in steady-state turnover conditions $E\text{-}Cu^{2+} = E\text{-}Cu^{+}$ is expected. Nevertheless, variable excesses of Cu^{2+} were observed, and in one study (1) only one copper of the two present in the dimeric enzyme molecule appeared to participate in the catalytic redox cycle, in spite of the established equivalence of the two copper binding sites (1, 3,4).

We have shown (5) that the nuclear spin of F^-, a very weak inhibitor of Cu, Zn SOD ($K \simeq 2M^{-1}$ ref. 6), is relaxed very efficiently and specifically by the Cu^{2+} at the active site of this enzyme. ^{19}F nmr relaxation was therefore used in the present work to probe the oxidation state of the active center of this enzyme during catalysis in the range of concentration used for enzyme assay, that means readily brought to steady state turnover by relatively low O_2^- concentrations.

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TABLE I

Reduction of Cu, Zn superoxide dismutase with sodium borohydride
The reaction was carried out in 0.05 M borate buffer pH 10.1, containing 2.5×10^{-4} M enzyme and 3 mM $NaBH_4$, and was stopped by addition of a concentrated solution of KH_2PO_4. The nmr measurements were performed after 1:25 dilution of the incubation mixture. The final pH was 5.8 and in these conditions the samples could be kept in air for long time without appreciable reoxidation.

Reaction time (sec)	Esr-detectable Cu %	$^{19}F^-$ relaxivity	
		$1/T_{1_p}$ (sec^{-1})	%
0	100	63.0	100
20	77	47.6	76
40	58	35.2	56
67	36	22.2	35
97	22	14.7	23
300	8	6.4	10

MATERIALS AND METHODS

Analytical grade reagents were used. Bovine erythrocyte SOD was prepared as previously described (7) and was assayed polarographically (8). Xanthine oxidase from milk was obtained from Boehringer. Cytochrome c, type III, was obtained from Sigma, as was catalase from bovine liver. Catalase was then freed of SOD as previously described (8).

Optical absorption measurements were carried out with a Varian Model 635 Spectrophotometer. Esr spectra were recorded at approximately 9 GHz with a Varian E-9 instrument. Nmr relaxation of ^{19}F nucleus were carried out and expressed as relaxivity (R) as previously described (5). The error limit of the nmr measurements was, in the experimental conditions used, 1.5%.

RESULTS

Cu, Zn SOD was found by both esr and ^{19}F nmr relaxation to be easily reduced by $NaBH_4$ at pH 10. The enzyme retained its full activity after this treatment. The esr-detectable copper and the corresponding ^{19}F R of samples of SOD reduced to variable extent showed an almost perfect correlation between each other (Table I). In order to study the ^{19}F R of O_2^--reduced SOD, two different systems were used for catalytic generation of O_2^-, namely an enzymatic one, xanthine-xanthine oxidase, and a chemical one, NADH-phenazine methosulphate (PMS). The latter system (9) was tested to verify the results obtained with the former one, which is likely to give rise to higher yields of secondary

TABLE II

Contribution of resting oxidized and working Cu, Zn bovine superoxide dismutase to nmr relaxation of $^{19}F^-$.

Xanthine	Xanthine oxidase	Time after addition of xanthine plus xanthine oxidase	$^{19}F^-$ relaxivity	
			$1/T_{1p}$ (sec^{-1})	% of the value measured in the resting enzyme)
-	-	-	4.80	100
+	-	20 min	4.75	99.4
-	+	20 min	4.89	101.9
+	+	10 min	2.74	57.1
+	+	20 min	2.69	56.1
+	+	1 h	2.71	51.5
+	+	2 h	2.75	57.3
+	+	24 h	4.76	97.0
+	+[a]	15 min	2.73	57.0
+	+[b]	15 min	2.77	57.8
+	+[c]	15 min	2.68	55.9

a) superoxide dismutase was reduced with $NaBH_4$.
b) without catalase.
c) in the presence of 10^{-2}M mannitol.

radicals (10). The production of O_2^- followed through the reduction of Fe(III)-cytochrome c at 547 nm (11).

a) xanthine-xanthine oxidase (Table II).

In a solution of 30 mM phosphate buffer, pH 7.4, containing $5x10^{-8}$M xanthine oxidase, $3x10^{-4}$M xanthine, $2x10^{-8}$M catalase, $3x10^{-5}$M EDTA, $1x10^{-4}$M ferricytochrome c and 0.5 M NaF, the absorbance at 547 nm increased at the constant rate of 0.019 min^{-1} which corresponds to an O_2^- production rate of 1.2 uM min^{-1} (12). $8x10^{-7}$M SOD was added in the place of cytochrome c in 10 separate experiments, using different enzyme batches for each. The F^-R value decreased to 55-60% of the value measured in the presence of the resting oxidized enzyme alone. Same experiment was repeated 5-6 times and the standard deviation resulted as 2%. A turnover rate of 0.75 O_2^-/Cu/min was calculated from the parallel cytochrome c experiment. The residual R was found to be independent of time between 10 min and 2 h from the start of O_2^- production and returned back to the value of the oxidized enzyme after 24 h. The same R was reached when the fully reduced enzyme (see Table I), was allowed to go to the steady-state in the same conditions. Catalase was added to avoid reduction and inactivation of SOD by H_2O_2 (13). No decrease of the superoxide dismutase activity was observed at

TABLE III

Effect of pH and F^- concentration on ^{19}F relaxivity of superoxide dismutase.
Conditions as for the experiments of Table II.
The relaxivity of the enzyme at steady-state was measured after 15 minutes from the addition of xanthine and xanthine oxidase.

pH	F^- (M)	^{19}F relaxivity (% of that of the oxidized enzyme)
5.6	0.5	59
6.5	0.5	58
7.35	0.5	63
8.6	0.5	60
10.2	0.5	62
7.35	0.1	53
7.35	0.2	57

any incubation time, and, in the absence of catalase, H_2O_2 effects were not detectable up to 20 min reaction time. Mannitol, a well known scavenger of $OH^\cdot$ radicals, was without effect.

b) NADH-PMS

A reaction mixture containing 2×10^{-4}M NADH, 1.2×10^{-5}M PMS and 0.5 M KF in 30 mM phosphate buffer, pH 7.4, produced O_2^- at an initial rate 1.2×10^{-5}M min^{-1}. When $\simeq$ 1 μM SOD was added, its F^- R reached the constant value of 56 $\pm$ 2% of that of the oxidized enzyme within 5 min. NADH alone reduced R down to practically zero in a much slower reaction.

A good correlation was found between the F^- R and the esr-detectable copper of samples of superoxide dismutase kept in steady-state turnover by NADH-PMS. In these experiments 0.1 mM SOD was used and the steady-state was established by 5 mM NADH + 2×10^{-5}M PMS, in the presence of 0.3 μM catalase. Low temperature esr spectra were recorded after 10 and 15 min incubation, and displayed 50$\pm$ 5% intensity loss with respect to controls. At both times the PMS radical was clearly detectable with the same intensity. In the presence of 0.5 M F^-, the esr intensity of the copper signal at steady-state was 58 $\pm$ 5% of the control.

No significant pH effects on the steady-state R were observed in the range pH 5.5-10.2. A small but significant decrease of the steady-state R was measured as the F^- concentration was decreased from 0.5 to 0.1 M (Table III). The steady-state R value does not depend on turnover rates ranging between 0.09 to 40 mole

O_2^-/Cu/min, which were obtained by changing the rate of O_2^- generation and/or the SOD concentration. Storage conditions and repeated freezing and freeze-drying did not change the catalytic constant and the R of the oxidized enzyme but affected the R ratio between the working and resting enzyme. This value increased from 55 $\pm$ 2% to 75 $\pm$ 2% after repeated freezing-thawing and to 70 $\pm$ 2% after either freeze-drying or storage at -20°C for several weeks, or standing at room temperature for several days.

DISCUSSION

The close correlation between the esr-detectable copper and the ^{19}F R of samples of Cu, Zn SOD reduced to different extent by $NaBH_4$ (Table I) demonstrates that the ^{19}F nmr relaxation method allows to estimate the Cu^{2+}/Cu^{+} ratio of the enzyme, provided the total protein concentration is known, with good precision (±2%) and much greater sensitivity (0.5-1 uM) than by esr (0.1 mM). Since exposure of the enzyme to fluxes of O_2^- generated by either enzymatic or chemical sources halves the ^{19}F R and the esr signal intensity of the resting enzyme, it follows that $Cu^{2+} = Cu^{+}$ in steady-state turnover conditions. Different results by earlier studies (1,2) can be explained by the present finding that state Cu^{2+}/Cu^{+} ratio is sensitive to the previous storage of enzyme preparations, while the catalytic constant and the oxidized enzyme R were the same in either samples giving 50% or 75% at the steady-state. This behavior is difficult to explain and further investigation is in progress on it; it gives a reasonable clue to the early results (1,2) which were carried out with samples prepared elsewhere and subjected to shipping treatments, such as freezing-thawing and freeze-thawing. As a matter of fact, when freshly prepared enzyme was tested in one study (14), using rapid-freeze esr and KO_2 in DMSO as a concentrated O_2^- source to assure steady-state attainment, lower $[Cu^{2+}]$ values, in the 75-60% range, were observed.

Our Cu^{2+} steady-state value in the presence of 0.5 M F^- was always significantly more than the theoretical 50% value (i.e. $\simeq$ 55%), but approached that value as the F^- concentration was lowered (Table III). As F^- is a competitive inhibitor of the enzyme (6), higher F^- concentrations increase the fraction of the inactive F-enzyme complexes and therefore of the Cu^{2+} present at the steady-state. From the stability constant value (6) it can be calculated

that $[F^-] \leq 0.02$ M should not affect the fraction of Cu^{2+}-enzyme present at the steady-state. This condition can be easily met with higher magnetic fields (6).

A final consideration concerns the observation that the Cu^{2+}, and consequently, the Cu^+ present at the steady-state is independent of turnover rate. In some turnover conditions the average life time of the Cu^+-enzyme resulted as long as 10 min. This implies that the reoxidation by O_2 of Cu^+-SOD formed in turnover is a very slow process at pH 7.4, as also shown by the experiments of reduction with borohydride (Table I).

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