Preparation of selectively metal-free and metal-substituted derivatives by reaction of Cu–Zn superoxide dismutase with diethyldithiocarbamate

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Incubation of Cu–Zn superoxide dismutase with diethyldithiocarbamate at increasing ligand/protein ratios and subsequent high-speed centrifugation led to proportional removal of copper from the protein, at variance with previous results [Misra (1979) *J. Biol. Chem.* **254**, 11623–11628]. No zinc was lost, even at very high excesses of chelating agent. In this way a copper-free protein could be readily prepared, with avoidance of the critical pH condition and the dialysis step required in a previous method employing cyanide. The holoprotein was fully reconstituted from the copper-free protein by stoichiometric re-addition of copper. From the mixture of metal-depleted forms originated by treatment with slight diethyldithiocarbamate excess, the protein containing copper only on one subunit, $[\text{Cu}_1\text{--Zn}_2]$, could be isolated by preparative column electrophoresis. This species reproducibly showed 25% more specific activity (catalytic constant per copper) than that of the native or reconstituted $[\text{Cu}_2\text{--Zn}_2]$ protein. This may result from long-range conformational effects between the active sites. By adding Co^{2+} ions to the vacant copper site of $[\text{Cu}_1\text{--Zn}_2]$ a hybrid molecule containing Cu(II) on one subunit and Co(II) in the homologous site of the other subunit was prepared. Its activity, referred to copper, was identical with that of the native protein.

The most active superoxide dismutase of eukaryotic organisms is a dimer of M_r 32000 containing one copper atom and one zinc atom on each identical subunit, $[\text{Cu}_2\text{--Zn}_2]$. Derivatives of this enzyme selectively deprived of one of either metal have been a major object of studies of the two metal-binding sites. The procedures available for the preparation of such derivatives are time-consuming and involve critical conditions to discriminate between the two metal ions. They are in fact dependent on reaction of two rather indiscriminate metal ligands, namely EDTA (in acid solution) and cyanide, and preparation of copper-free (Rotilio *et al.*, 1972), zinc-free (Beem *et al.*, 1974) or fully metal-free (McCord & Fridovich, 1969) apoprotein relies on careful pH variations in the removal or reconstitution steps. More recently diethyldithiocarbamate has been recognized as a further reagent for the superoxide dismutase copper (Misra, 1979). That study, however, gave no indications on the final products of its reaction with the protein, and in

particular was negative as regards the possibility of obtaining copper-free superoxide dismutase.

In the present paper we describe the conditions for preparing from Cu–Zn superoxide dismutase that has reacted with diethyldithiocarbamate a copper-free protein capable of reconstitution to the native protein on stoichiometric addition of copper. The isolation and characterization of the protein with copper bound to only one subunit, $[\text{Cu}_1\text{--Zn}_2]$, is also reported, with reference to the problem of interaction between active sites on different subunits in the catalytic mechanism of the enzyme (Fielden *et al.*, 1974; Rotilio *et al.*, 1977). The cobalt–copper hybrid, $[\text{Cu}_1\text{Co}_1\text{--Zn}_2]$, having one subunit active ($[\text{Cu}\text{--Zn}]$) and the other with the copper replaced by the inactive cobalt ($[\text{Co}\text{--Zn}]$), could also be obtained from the $[\text{Cu}_1\text{--Zn}_2]$ derivative.

Materials and methods

Reagent-grade chemicals were used without further purification.

Cu-Zn superoxide dismutases were isolated from ox, horse or pig erythrocytes and from yeast by slight modifications of current methods (McCord & Fridovich, 1969; Goscin & Fridovich, 1972). In particular, the supernatant from the chloroform/ethanol precipitation was treated with lead acetate (22.5 g/l), and the resulting precipitate was dissolved in 0.33 M-potassium phosphate buffer, pH 6.0. For the pig enzyme the dissolved precipitate was dialysed against 5 mM-Tris/HCl buffer, pH 9.0, and then applied to a QAE-Sephadex A-50 column (2.5 cm $\times$ 30 cm) equilibrated with the same buffer. Elution was performed with a linear pH gradient between pH 9.0 and pH 5.0, 5 mM-Tris/HCl buffer and sodium cacodylate/HCl buffer being used. For the ox, horse and yeast enzymes, the dissolved precipitates were dialysed against 20 mM-sodium cacodylate/HCl buffer, pH 7.4, and then applied to a QAE-Sephadex A-50 column (2.5 cm $\times$ 30 cm) equilibrated with the same buffer. Elution was performed with a linear pH gradient between pH 7.4 and 5.0, 20 mM-sodium cacodylate/HCl buffer being used. Cobalt derivatives of superoxide dismutase were obtained as described by Calabrese *et al.* (1976, 1979). The enzyme activity was assayed polarographically (Rigo *et al.*, 1975). Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis (1964), with some modifications (Rigo *et al.*, 1977a). Gels were stained either for the protein with Amido Black (Davis, 1964) or for the enzyme activity as described by Beauchamp & Fridovich (1971). Densitometric analysis of the electrophoretograms was performed with a CGA Cello-matic densitometer equipped with a CGA AT3 recorder. Optical spectra were recorded on a Beckman UV 5230 spectrophotometer. E.p.r. spectra were recorded with a Varian model E-9 spectrometer. Metal analyses were performed by atomic-absorption spectrometry with a Hilger and Watts model H-1170 instrument.

Results

Copper removal from bovine superoxide dismutase by diethyldithiocarbamate

It has been reported (Misra, 1979) that diethyldithiocarbamate forms with superoxide dismutase a diethyldithiocarbamate-copper complex, detectable by its absorption maximum at 450 nm, which cannot be separated from the protein by either dialysis or gel filtration. The following procedure was found to be suitable for a complete separation of the complex from the protein. To protein solutions in the 0.1–0.5 mM concentration range, buffered with 0.1 M-potassium phosphate, pH 7.4, diethyldithiocarbamate was added in various proportions. The mixture was incubated at 37°C until no further increase of the 450 nm band was observed (approx.

2 h at a [diethyldithiocarbamate]/[copper] ratio of 5:1). The yellow solution was then centrifuged at 39 000 g for 30 min, and the colourless supernatant was then exhaustively dialysed against the phosphate buffer.

Fig. 1 shows the electrophoretic pattern of samples treated with increasing [diethyldithiocarbamate]/[copper] ratios, after staining either for the protein (Fig. 1a) or for the superoxide dismutase activity (Fig. 1b). The native enzyme shows the typical multiple pattern of active bands, already established (Malinowski & Fridovich, 1979) to be electrophoretic forms of the protein with the same metal content. The data in Table 1, relative to the

Fig. 1. Electrophoretic pattern of bovine superoxide dismutase treated with increasing [diethyldithiocarbamate]/[copper] ratios

For experimental details see the text. [Diethyldithiocarbamate]/[copper] ratios were: (i) 0; (ii) 0.5:1; (iii) 1:1; (iv) 2:1; (v) 5:1. In panel (a) gels were stained with Amido Black for protein: 30 μ g of protein was applied to the gels. In panel (b) gels were stained for enzyme activity: 30 ng of protein was applied to the gels.

Table 1. Chemical, catalytic and electrophoretic parameters of Cu-Zn superoxide dismutase treated with increasing [diethyldithiocarbamate]/[copper] ratios

For experimental details see the text. The fourth column represents the difference between the initial copper concentration and the amount of copper removed by diethyldithiocarbamate, as monitored by the absorption at 450 nm of the diethyldithiocarbamate-copper complex (Misra, 1979). Abbreviation used: DDC, diethyldithiocarbamate.

[DDC]/[Cu] ratio	Residual Cu (% e.p.r. signal intensity)	Residual Cu (% absorption at 680 nm)	Residual Cu (%) ([Cu _{holo}]— [Cu-DDC ₄₅₀])	Residual activity (%)	Electrophoretic bands (%)		
					Slowest	Intermediate	Fastest
0	100	100	100	100	90.9	9.1	
0.5	85	82	84	83	60.4	37.5	2.3
1	70	68	69	60	43.5	43.7	12.9
2	40	40	38	47	38	43.6	18.5
5	3	2	0	10		9.7	90.3

Fig. 2. Optical (a and b) and e.p.r. (c) spectra of bovine superoxide dismutase treated with diethyldithiocarbamate at a [diethyldithiocarbamate]/[copper] ratio of 5:1 and of the reconstituted protein

Holoprotein (5.3 mM) in 0.1 M-phosphate buffer, pH 7.4, was treated with 53 mM-diethyldithiocarbamate in the conditions described in the text. Trace A, native enzyme; trace B, after incubation with diethyldithiocarbamate and further treatments (see the text); the broken trace is the spectrum of the protein corresponding to trace B after addition of a stoichiometric amount of copper.

same experiments, show that with increasing [diethyldithiocarbamate]/[copper] ratios there is a parallel decrease of copper content and superoxide dismutase activity. In particular, a [diethyldithiocarbamate]/[copper] ratio of 5:1 led to nearly full removal of the copper from the protein. No zinc was lost in the same conditions; in fact, no difference of the zinc content was found before and after diethyldithiocarbamate treatment, as measured by atomic-absorption spectroscopy. The electrophor-

etic pattern can thus be explained by the simultaneous presence at [diethyldithiocarbamate]/[copper] ratios below 5:1, of the active holoprotein, the inactive fastest copper-free protein and an intermediate active band, very probably the dimer with one subunit devoid of copper.

Fig. 2 shows that the protein fully depleted of copper by treatment at a [diethyldithiocarbamate]/[copper] ratio of 5:1 was able to reconstitute to the holoprotein by incubation with stoichiometric

copper. Also, the enzyme activity (more than 90%) and the native electrophoretic mobility were completely recovered.

Effect of diethyldithiocarbamate on zinc and cobalt in the bovine enzyme

In the conditions used for removal of copper, no zinc was lost from the protein, as shown by atomic-absorption measurements. Much higher [diethyldithiocarbamate]/[copper] ratios (up to 100:1) and longer incubation times had no effect on the zinc content of either native or copper-free protein. To test whether this result was due to the metal-diethyldithiocarbamate affinity or to the accessibility of the second metal-binding site for the chelating agent, diethyldithiocarbamate was treated with two different cobalt derivatives of the bovine enzyme, namely the copper-free enzyme with cobalt in the place of the native zinc ($[\text{Co}_2]$) and the enzyme with the cobalt in the place of native copper, $[\text{Co}_2\text{-Zn}_2]$. In both cases a [diethyldithiocarbamate]/[cobalt] ratio of 10:1 led to full removal of the metal.

Metal removal by diethyldithiocarbamate from superoxide dismutase of different organisms

The copper-free protein was obtained with iden-

tical modality and results from human, horse and pig Cu-Zn superoxide dismutase. The reaction of diethyldithiocarbamate with the yeast enzyme was much faster, although the copper lost, at the end of reactions with different [diethyldithiocarbamate]/[copper] ratios, was approximately the same as for other proteins. Full copper removal by diethyldithiocarbamate was obtained by centrifuging the incubation mixtures at a [diethyldithiocarbamate]/[copper] ratio of 4:1 only 60 min after addition of the reagent at 37°C. No zinc was lost in any conditions by all the enzymes tested, as untreated and diethyldithiocarbamate-treated samples were found to have the same [zinc]/[protein] value by atomic-absorption measurements.

Isolation and characterization of the $[\text{Cu}_1\text{-Zn}_2]$ derivative

In the [diethyldithiocarbamate]/[copper] ratio range between 2:1 and 1:1 (Fig. 1) the major component of the electrophoretic pattern is the intermediate band between the holoenzyme and the copper-free superoxide dismutase. This component was separated from the mixture by preparative column electrophoresis on Sephadex G-25 by the procedure of Whitehead *et al.* (1971), under conditions of analytical gel electrophoresis (Rigo *et al.*,

Fig. 3. Elution and electrophoretic patterns of column electrophoresis on Sephadex G-25 for the isolation of the $[\text{Cu}_1\text{-Zn}_2]$ protein

The elution pattern is expressed as the A_{260}/A_{280} ratio of the fractions (see the text). In the upper part the electrophoretic patterns of the fractions marked by arrows are reported. Outside the frame the electrophoretic pattern of the starting material (see the text) is reported.

1977a). As the holoenzyme and the copper-free superoxide dismutase have different u.v. absorbances (Rotilio *et al.*, 1972), the elution from the Sephadex G-25 column was followed by recording the absorbances at 260 and 280 nm (Fig. 3). The fractions having A_{260}/A_{280} ratio 1.4 turned out to contain only the electrophoretic band with intermediate mobility (Fig. 3). These fractions were pooled, and their copper content was determined to be 1 atom per 32000 daltons by atomic-absorption measurements and by double integration of the e.p.r. signal. Optical and e.p.r. spectra of the $[\text{Cu}_1\text{-Zn}_2]$ species were the same as those of the native $[\text{Cu}_2\text{-Zn}_2]$ protein. Its electrophoretic pattern was stable on repeated analyses during prolonged standing at room temperature (up to 3 days). By addition of complementary copper amounts the $[\text{Cu}_1\text{-Zn}_2]$ protein could be reconverted into the native protein as judged by e.p.r. and optical spectra and by electrophoretic mobility.

Preparation of the $[\text{Cu,Co-Zn}_2]$ hybrid

It has been shown (Calabrese *et al.*, 1979) that cobalt, in particular conditions of pH and buffering ions, binds to copper-free superoxide dismutase at the copper-binding site of the native enzyme. In the same conditions 0.8 equiv. of Co(II) was added to the $[\text{Cu}_1\text{-Zn}_2]$ protein. The metal content of this derivative, after extensive dialysis, was 1 copper atom and 0.7 cobalt atom per protein molecule, as measured by atomic absorption and by absorption coefficients at 680 and 605 nm (Calabrese *et al.*,

Table 2. *Catalytic constants of superoxide dismutase derivatives with different metal content, as measured by polarography*

Superoxide dismutase derivative	$10^9 \times k/\text{copper}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Native protein	0.85
$[\text{Cu}_1\text{-Zn}_2]$	1.05
$[\text{Cu}_1\text{-Zn}_2] + \text{Cu}^*$	0.84
$[\text{Cu}_1\text{-Zn}_2] + \text{Co}^\dagger$	0.82

* $[\text{Cu}_1\text{-Zn}_2]$ protein reconstituted by addition of the copper complement (Rotilio *et al.*, 1972).

† $[\text{Cu}_1\text{-Zn}_2]$ protein reconstituted by addition of cobalt as described in the text.

1979). Fig. 4 shows the optical spectrum of $[\text{Cu,Co-Zn}_2]$ protein and other Co(II) derivatives.

Catalytic properties of the $[\text{Cu}_1\text{-Zn}_2]$ protein and of the $[\text{Cu,Co-Zn}_2]$ hybrid

Table 2 reports the catalytic constants, k , of the derivatives obtained in the present work, as measured by polarography (Rigo *et al.*, 1975). The $k/\text{Cu(II)}$ value of the $[\text{Cu}_1\text{-Zn}_2]$ protein was reproducibly (three different preparations) higher than that of either the native $[\text{Cu}_2\text{-Zn}_2]$ protein or the $[\text{Cu,Co-Zn}_2]$ hybrid.

Discussion

The results reported in the present paper show that, at variance with previous results (Misra, 1979), the diethyldithiocarbamate-copper complex of superoxide dismutase can be separated from the protein. Therefore diethyldithiocarbamate can be used to obtain, by a rapid and easy procedure, a copper-free superoxide dismutase. Furthermore the data in Fig. 1 and Table 1 demonstrate that the diethyldithiocarbamate-copper complex can be separated from the protein even at low $[\text{diethyldithiocarbamate}]/[\text{copper}]$ ratios. This conclusion is hardly compatible with the suggestion (Misra, 1979) that a ternary diethyldithiocarbamate-copper-protein complex is formed with low $[\text{diethyldithiocarbamate}]/[\text{copper}]$ ratios, and rather indicates that displacement of the copper from the protein occurs at any diethyldithiocarbamate concentration, although it is true that the diethyldithiocarbamate-copper complex adheres tenaciously to the protein.

The fact that diethyldithiocarbamate, even at very high concentrations, is unable to remove the zinc from either the holoprotein or the copper-free protein, though it is able to remove cobalt from the superoxide dismutase derivative where it is bound at the zinc site, indicates that diethyldithiocarbamate

Fig. 4. *Optical spectra of cobalt superoxide dismutase derivatives*

Trace A, $[\text{Cu}_1\text{-Zn}_2]$, 0.9 mM copper; trace B, $[\text{Cu,Co-Zn}_2]$, 0.9 mM copper and 0.63 mM cobalt; trace C, $[\text{Cu}_2\text{-Co}_2]$, 0.9 mM copper and 0.65 mM cobalt. The broken trace is the spectrum of sample corresponding to trace C after reduction of copper with H_2O_2 .

has access to the zinc-binding site but that it cannot form a diethyldithiocarbamate-zinc complex separable from the protein in the experimental conditions suitable for removal of other metals.

Since progressive copper depletion was obtained with increasing [diethyldithiocarbamate]/[copper] ratios (Table 1), the isolation of the $[\text{Cu}_1\text{-Zn}_2]$ protein was successfully attempted, with a view to the question concerning the relationship between dimeric structure and catalytic function of superoxide dismutase (Fielden *et al.*, 1974; Rotilio *et al.*, 1977). Optical and e.p.r. spectra of this derivative confirm the spectroscopic equivalence of the two copper-binding sites (Fielden *et al.*, 1974). The $[\text{Cu}_1\text{-Zn}_2]$ protein represents the ideal case of a 'hybrid' between active and inactive monomers, as it can be obtained without strong treatments (Malinowski & Fridovich, 1979). It is apparently stable, as no metal migration from the occupied to the vacant subunit could be detected by electrophoresis after 3 days' standing at room temperature. Measurements of the catalytic constants show that the enzyme activity, as referred to copper, decreases with filling of the second copper-binding site with either copper or cobalt (Table 2). These results are consistent with previously reported results (Rigo *et al.*, 1977b) on reconstitution of the copper-free enzyme with increasing amounts of copper, i.e. in conditions where mixtures of the various forms indicated in Fig. 1 were present. They also may explain the data of Table 1, where enzyme samples having 3% or less of residual copper retain 10% of activity. In this case, the very low values of either parameter do not allow a quantitative relationship; however, they may indicate that copper-depleted molecules have higher activity. A possible explanation of this fact is that binding of copper at the copper-binding site of a monomer may affect the site on the other monomer as to decrease its catalytic constant with no significant changes of its spectroscopic properties. The nature of this change is still obscure.

Further information on the interaction between copper-binding sites of different subunits can be obtained with the $[\text{Cu,Co-Zn}_2]$ hybrid. It is a spectroscopic hybrid as well, which is potentially able to record information transfer between the same sites on different monomers.

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