Re-examination of the Reaction of Diethyldithiocarbamate with the Copper of Superoxide Dismutase*

Dina Cocco‡, Lilia Calabrese‡, Adelio Rigo§, Emanuele Argesë§, and Giuseppe Rotilio‡

From the ‡Institute of Biological Chemistry and Consiglio Nazionale delle Ricerche Center for Molecular Biology, University of Rome, Rome, Italy and the §Institute of Physical Chemistry, University of Venice, Venice, Italy

(Received for publication, August 12, 1980, and in revised form, April 28, 1981)

The reaction of the copper of (Cu,Zn)-superoxide dismutase with diethyldithiocarbamate was studied at pH = 7.4 and the results obtained led to a reaction scheme basically different from the conclusion of a previous study (Misra, H. P. (1979) J. Biol. Chem. 254, 11628). The analysis of optical and ESR spectra at 9 and 35 GHz, at different ligand/protein ratios and reaction times, showed that a ternary diethyldithiocarbamate-Cu(II)-protein complex never formed in spectrosopically detectable amounts. The system is described in any condition as the mixture, in variable proportions, of only two components, that is the diethyldithiocarbamate-free Cu(II) chelate and the copper-depleted protein. The formation of a catalytically active copper-diethyldithiocarbamate intermediate with distinct optical and ESR spectra was also ruled out by kinetic studies, which demonstrated that enzyme inactivation strictly parallels the binding of diethyldithiocarbamate as monitored by optical absorption and ESR. Separation of the copper complex from the protein was obtained for the first time, and the procedure was suitable for rapid preparation of reconstitutable copper-free superoxide dismutase.

Reversible inactivation of (Cu,Zn)-superoxide dismutase by diethyldithiocarbamate has been shown to occur in vivo (1) and in vitro (2). Involvement of copper has been demonstrated by reactivation and spectral evidence (2). In those investigations, formation of an enzyme-bound Cu(II)·DDC complex was emphasized. Moreover, it was concluded that such a complex is catalytically active and enzyme inactivation occurs upon binding of a second DDC molecule which displaces the copper from the enzyme (2).

However, the statement that a stable and active enzyme-Cu-DDC complex was formed at low DDC/enzyme ratio relied on interpretation of ESR spectra and enzymic assays that were intrinsically liable to alternative explanation. A second intriguing result of this early work was that separation of an apoprotein was not achieved. In the light of these facts, the reaction of DDC with (Cu,Zn)-superoxide dismutase was reinvestigated. A clean separation of Cu·DDC complex and copper-free enzyme was obtained. As far as the nature and the catalytic activity of the intermediate complex are concerned, different conclusions were reached on the basis of more extensive spectral and kinetic analysis of the system.

MATERIALS AND METHODS

Superoxide dismutase was isolated from bovine erythrocytes as previously described (3) and was assayed polarographically at pH 9.2 (4) or by 51Fe relaxivity (5) at pH 7.4. The copper-free protein was prepared as previously described (6). DDC was obtained from Merck and was used without further purification. Optical spectra were recorded on a Beckman spectrophotometer UV 5230. ESR spectra, at approximately 9 and 35 GHz, were obtained with a Varian model E-9 spectrometer equipped with the Varian variable temperature accessory. Metal analysis were performed by atomic absorption spectrometry with a Hilger and Watts Atomspek, model H 1170.

RESULTS

ESR Spectra as a Function of the DDC/Protein Ratio and the Incubation Time—Fig. 1A reports the ESR spectra of incubation mixtures where increasing equivalents of DDC were reacted with the protein until further spectral changes were practically not appreciable. The reaction was followed optically at 450 nm at pH 7.4 as previously described (2). The ESR spectra of Fig. 1A show a continuous variation of line shape throughout the full range of DDC concentrations used, with appearance of at least two isosbestic points. Identical results were obtained when the reaction was followed as a function of time (Fig. 1B). In both cases the line shape changes can be described in terms of only two component spectra, that is, the spectrum of the native enzyme and the sharp isotropic signal around g = 2 with no hyperfine structure, already shown to be (2) the DDC complex of free copper.

This analysis was confirmed by measurements at higher frequency on samples incubated in the presence of low DDC/protein ratio (Fig. 2). They unequivocally show that no other species is detectable in the spectral envelope but the native enzyme and a novel sharp signal now resolved as to give two very close g values. The spectrum obtained by Misra (2) in the same conditions and attributed to a ternary DDC-Cu-protein complex with hyperfine structure substantially identical with that of the native enzyme is therefore a composite one. In particular it consists of the residual native spectrum and of a novel signal which can be identified as that of the DDC-Cu chelate. In fact Fig. 2 (inset, lines b and c) shows that the spectrum arising from the incubation of the native enzyme with DDC is identical with the spectrum obtained by adding a comparable amount of free DDC-Cu to the holoprotein.

Separation of the Copper from the Enzyme as its DDC Complex and Preparation of Copper-depleted Superoxide Dismutase—A major argument to support the idea of a copper-DDC complex still bound at the native copper binding site, which formed when the enzyme was exposed to low concen-
FIG. 1. Effect of DDC on ESR spectrum of superoxide dismutase. A, 0.7 mM enzyme in 100 mM potassium phosphate buffer, pH 7.4, was incubated with increasing amount of DDC for 96 h at 20°C. Line a, enzyme alone; line b, enzyme plus 0.7 mM DDC; line c, enzyme plus 1.4 mM DDC; line d, enzyme plus 2.8 mM DDC; line e, enzyme plus 7 mM DDC. B, 1 mM enzyme in 100 mM potassium phosphate buffer, pH 7.4, was incubated at 20°C with 10 mM DDC and at intervals was frozen in liquid nitrogen. Line a, native enzyme; line b, recorded after 300 min incubation; intermediate curves, recorded after 15, 45, and 150 min (bottom to top). ESR spectra were recorded at 9.14 GHz frequency, 20 mW power, 10 G modulation amplitude, and -160°C sample temperature.

tration of DDC was that the complex could neither be extracted by organic solvent nor lost upon dialysis or gel filtration through a Sephadex G-75 column (2). These results were confirmed by us. However, high speed centrifugation was capable of separating a yellow precipitate from the protein with consequent removal of copper. Fig. 3 (lines a–e) shows the optical and ESR spectra of protein samples reacted with increasing amounts of DDC for 96 h at 20°C and then centrifuged at 39,000 × g for 20 min. Five excesses of DDC were able to remove all the copper under the conditions used. It is apparent (see Fig. 3C) that there is a linear relationship between the copper loss and the DDC/Cu ratio at least up to DDC/Cu = 2. Obviously the rate of attainment of such a situation critically depended on absolute concentrations. Readdition of the relative copper complements to the copper-deprived sample led to full reconstitution of the native protein (Fig. 3, A and B, dotted lines).

Effect of the Protein on the Physical State of the Cu-DDC Complex—The yellow precipitate is likely to be a polymeric form of the Cu-DDC complex. An interesting point which

FIG. 2. ESR spectra at 35 GHz of superoxide dismutase treated with DDC. One mM enzyme in 100 mM potassium phosphate buffer, pH 7.4, was incubated at 20°C for 96 h with 2 mM DDC. Line a, enzyme alone; line b, enzyme plus DDC. The inset shows the central part of spectrum b at reduced gain (x 0.1); line c, spectrum of the aqueous DDC-Cu chelate (0.6 mM CuSO₄ and 1.2 mM DDC) taken in the presence of 0.5 mM holoenzyme. The spectra were recorded at 35.41 GHz frequency, 20 mW power, 10 G modulation amplitude and -120°C sample temperature.
may explain the previous interpretation (2) that the precipitate obtained from the DDC-protein reaction after high speed centrifugation could not be resuspended in buffer just as the complex between free Cu(II) and DDC could not, even though the protein/DDC incubation mixtures did not show insoluble material at any time or ligand concentration. This contradictory result was found to be related to an effect of the protein moiety of superoxide dismutase on the physical state of the Cu-DDC complex leading to a better solubilization of it in water. Either holo- or copper-free samples had the same effect. Some related ESR spectra are shown in Fig. 4 and are evidently identical with those obtained in the reaction of the holoprotein with DDC (see Figs. 1 and 2).

**Kinetic Studies of the Reaction**—The previous results are not in accord with the reported observation (2) that superoxide dismutase retained essentially full activity upon incubation with modest molar excesses of DDC. In order to explain this contradiction, further analysis of the time course of the reaction and of its kinetic parameters was carried out at pH 7.4. Fig. 5 shows that the increase of absorbance at 450 nm due to formation of Cu-DDC complex occurs in two steps (A and B), both having first order kinetics. Step A is much faster \( k_A / k_B \approx 10 \) and accounts for approximately 85% of the total absorbance.

It can be described by an equation of the type:

\[
\frac{d(A_{450\text{ nm}})}{dt} = k'\text{[enzyme][DDC]}^{0.5}
\]

where \( A_{450\text{ nm}} \) is a linear function of the Cu-DDC complex concentration, with \( k' = 220 \pm 30 \text{ min}^{-1} \text{M}^{-0.5} \).

Fig. 6 shows the time course of the reaction in terms of both formation of the 450-nm band and changes of enzyme activity. It is evident that the activity loss closely parallels the step A of the absorbance changes. This pattern was observed at all DDC/protein ratios and in particular when 0.1 mM enzyme was reacted with slightly more than stoichiometric DDC (see Fig. 7 of Ref. 2).

**DISCUSSION**

The reaction of DDC with copper of superoxide dismutase is a relatively slow reaction which apparently leads to a cooperative binding of two DDC molecules/copper with consequent removal of the metal from the protein at any DDC/protein ratio. This conclusion is supported by the absence of any spectral intermediate imputable to DDC-Cu-protein...
complex neither at early reaction times nor in the presence of low molar excesses of DDC. This was verified by both optical and ESR spectra. In fact, only the 450-nm band, typical of the complex between DDC and free Cu, is observed during the reaction in any condition. In the case of Cu-carbonic anhydrase the formation of a ternary DDC·Cu·protein complex is monitored by the presence of an absorption band at shorter wavelengths. The slightly different extinction coefficients previously measured (2) for the free Cu(II) and the protein-bound Cu upon reaction of DDC, as well as the failure to remove the complex by dialysis, gel filtration, or solvent extraction, can be accounted for by the interaction of Cu·DDC with the protein part of superoxide dismutase (Fig. 4). This interaction does not necessarily exclude the original copper binding site but does not involve coordination of copper with the native ligands. This is clearly demonstrated by the ESR spectra. The spectra obtained at early reaction times as well as at low DDC/Cu ratios were attributed (2) to a novel species formed by coordination of a single DDC molecule to the copper still attached at some of its native ligands. They consist, on the contrary, of two species in any condition, that is the native protein and the DDC·Cu chelate. The 35 GHz spectra (Fig. 2, line b) and the identical spectra obtained on addition of Cu·DDC to unreacted superoxide dismutase (Fig. 2, line c, and Fig. 4) are unequivocal evidence for this interpretation. On the other hand, the ESR parameters of Cu-carbonic anhydrase were modified by DDC coordination to the copper still bound to some ligands of the native binding site, and the observed changes were as expected in cases of mixed nitrogen/sulfur coordination.

Beside spectral evidence, an additional, and perhaps major argument in favor of the formation of the DDC·Cu complex, in any condition of time and ligand/protein ratio in the reaction of DDC with the superoxide dismutase copper, is that that complex could in fact be always removed from the protein by centrifugation. In line with this result, superoxide dismutase was shown to be inactivated by DDC at the expected extent and with a time course parallel to complex formation at any ligand excess. The apparently conflicting results of previous work (2) can be explained by inadequate control of experimental conditions, as the kinetic analysis reported in the present paper indicates that the measurements reported in Fig. 7 of Ref. 2 are far from the equilibrium conditions for low molar excesses of DDC, the absolute concentrations of the reagents being too low.

The unusual order of the reaction in the DDC (1.5) is not easy to explain and is object of further investigation. The slower and later phase of complex formation, found to be irrelevant to the inactivation process, is likely to reflect time-depending changes of the loose interaction between the protein and the Cu(II)·DDC complex. Slight turbidity is in fact observed in the reaction mixture after very long incubation times.

A promising result of this work is that copper-depleted superoxide dismutase can be easily and rapidly prepared by reaction with DDC. The reaction is in fact accelerated by heating or pH changes without alteration of the protein. This preparative aspect of the reaction of DDC with superoxide dismutase will be described elsewhere.

REFERENCES