Reconstitution of Cu,Zn-Superoxide Dismutase by the Cu(I)•Glutathione Complex*

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The reconstitution of Cu,Zn-superoxide dismutase from the copper-free protein by the Cu(I)•GSH complex was monitored by: (a) EPR and optical spectroscopy upon reoxidation of the enzyme-bound copper; (b) NMR spectroscopy following the broadening of the resonances of the Cu(I)•GSH complex after addition of Cu-free, Zn-superoxide dismutase; and (c) NMR spectroscopy of the Cu-free, Co(II) enzyme following the appearance of the isotropically shifted resonances of the Cu(I)•Co enzyme. Cu(I)•GSH was found to be a very stable complex in the presence of oxygen and a more efficient copper donor to the copper-free enzyme than other low molecular weight Cu(II) complexes. In particular, 100% reconstitution was obtained with stoichiometric copper at any GSH:copper ratio between 2 and 500. Evidence was obtained for the occurrence of a Cu(I)•GSH-protein intermediate in the reconstitution process. In view of the inability of copper-thionein to reconstitute Cu,Zn-superoxide dismutase and of the detection of copper•GSH complexes in copper-overloaded hepatoma cells (Freedman, J. H., Ciriolo, M. R., and Peisach, J. (1989) J. Biol. Chem. 264, 5598–5605), Cu(I)•GSH is proposed as a likely candidate for copper donation to Cu-free, Zn-superoxide dismutase in vivo.

Superoxide dismutases are metalloenzymes that play an essential role in the defense of the cell against potentially toxic derivatives of the biological activation of oxygen. They are ubiquitous enzymes; and in particular, the isoenzyme that carries one copper and one adjacent zinc ion in the catalytically active center on each of its two identical subunits is typical of, although not restricted to, eukaryotic cytosol (Banister et al., 1987). A still unresolved problem in the comprehension of the physiological regulation of its activity is the biological mechanism by which the catalytically active copper is taken up by the protein moiety in the active-site pocket. Previous evidence (Caas et al., 1979) points to a role of zinc in giving the adjacent copper-binding site the geometry that is typical of the native holoenzyme and consists of a tetrahedral copper donation to Cu-free, Zn-superoxide dismutase; and (c) NMR spectroscopy monitoring Cu(I)-protein binding. No transfer of copper to the copper-free enzyme was observed when the copper-glutathione complex was in the oxidized form Cu(II)•GSSG, whereas the reduced complex, Cu(I)•GSH, fully reconstituted the enzyme in a very efficient process apparently involving a Cu(I)-GSH-protein intermediate. This result is a strong indication that GSH may be able to donate Cu(I) to the copper-free enzyme in vivo.

EXPERIMENTAL PROCEDURES

All chemicals used were reagent-grade and were of the highest purity available from commercial sources. Glutathione (reduced or oxidized) was obtained from Boehringer Mannheim. L-Cysteine and L-methionine were from Sigma. CuCl was obtained from Aldrich. All stock solutions of buffers and other chemicals used were prepared with water pretreated with Chelex 100 (Bio-Rad) to remove traces of copper. Stock solutions of CuSO4 in double-distilled water were prepared shortly before use.

Cu,Zn-superoxide dismutase was isolated from bovine erythrocytes according to McCord and Fridovich (1969), and its concentration was calculated either by the absorbance of the copper chromophore (McCord and Fridovich, 1969) or by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The copper-cobalt derivative was prepared by addition of CoCl2 to the Zn-free enzyme which was obtained as previously described (Valentine et al., 1979). The final concentration of the cobalt bound to the enzyme was controlled spectrophotometrically (Calabrese et al., 1972). The copper-free derivatives of either the holo- or copper-cobalt enzymes were prepared by reducing the copper with excess potassium ferrocyanide and dialyzing for 12 h at 4 °C against 0.1 M phosphate buffer containing 0.05 M KCN at pH 6.0 (Rigo et al., 1977). The samples were further dialyzed for 24 h at 4 °C against water. Final copper content was less than 2%. The reduced Cu(I) form of the enzyme was prepared by an anerobic treatment with NaBH4 as previously described (Vigino et al., 1981). The enzyme activity was assayed with the polarographic method of Rigo et al. (1976).

The Cu(I)•GSH complex was prepared by adding an anerobic solution of GSH in 0.1 M phosphate buffer, pH 7.0, to an appropriate dose of Cu(I)•GSH in 0.1 M phosphate buffer, pH 7.0.
amount of CuCl powder in a Thunberg apparatus under nitrogen atmosphere. The CuCl powder was priorly weighted and introduced into the Thunberg cell in a dry box flushed with nitrogen. The final GSH:copper molar ratio was 0.5. Cu(I) complexes with GSH were also prepared in a way that may be considered as closer to physiological conditions, i.e. adding CuSO₄ to solutions of GSH in 0.1 M phosphate buffer, pH 7.0, either under strictly anaerobic conditions or in air. Several complexes with different GSH:copper ratios, starting from 3:1, were prepared in this way. However prepared, the Cu(I) complexes were devoid of Cu(II) as evaluated by EPR spectroscopy (<1%). An aliquot of the Cu(I)-GSH complex solution was then transferred by a gas-tight syringe to a Thunberg apparatus sealed to an EPR tube containing an anaerobic solution of Cu-free Zn-superoxide dismutase in 0.05 M phosphate buffer, pH 7.4. Under either aerobic or anaerobic conditions, a cuprous complex with cysteine was unstable at the same pH. Cu(II) complexes with GSSG or methionine were prepared in air by adding CuCl₂ to 3-fold excesses of the various complexing agents and were added to Cu-free Zn-superoxide dismutase in 0.05 M phosphate buffer, pH 7.4.

X-band EPR spectra were recorded at 100 K with a Bruker ESP-300 instrument operating at 9.42 GHz. EPR settings for the spectra were: modulation amplitude, 10 G; microwave power, 20 milliwatts; time constant, 164 ms; and scanning time, 42 s. Signal intensity was evaluated by double integration of the spectra against a Cu(II)-EDTA standard.

Reoxidation rates of the Cu(I)-GSH complex, Cu(I)-GSH-Cu-free, Zn-superoxide dismutase, and Cu(I),Zn-superoxide dismutase in 0.05 M phosphate buffer, pH 7.4, in air were measured spectrophotometrically following the increase of optical density at 625 nm, typical of the Cu(I)-GSSG complex (Postal et al., 1985), and at 580 nm, typical of Cu(II),Zn-superoxide dismutase (McCord and Fridovich, 1969). The reoxidation experiments were always carried out in the presence of 0.1 μM catalase to eliminate H₂O₂, a possible reaction product, which may interfere by both reducing and denaturing the enzyme (Bray et al., 1974; Hodgson and Fridovich, 1975). Optical spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer.

'H NMR spectra were recorded at 400 MHz in 20% deuteriated solutions of 0.05 M phosphate buffer, pH 7.4, with a Bruker AM-400 spectrometer. The spectra of GSH and the Cu(I)-GSH complex were recorded with solvent suppression by selective presaturation of 1.7 s of the water proton resonance. 128 decays were accumulated on a spectral width of 6 kHz using a time domain of 8,000 data points. Cu(II) and Cu-free, Zn-superoxide dismutase were added in minute amounts directly to the NMR tube. The spectra of the isotropically shifted resonances of the Cu,Co-superoxide dismutase (Banci et al., 1987) were obtained by using a modified pulse sequence (Hochmann and Kellerhals, 1980) to suppress H₂O and bulk protein signals. The spectra consisted of 10,000 scans with 8,000 data points and a bandwidth of 166 kHz. Chemical shifts were measured from H₂O which was assumed to be 4.8 ppm from tetramethylsilane. NMR peaks were labeled on the spectra of the Cu(I),Co protein with capital letters in alphabetical order from higher parts/million values.

RESULTS AND DISCUSSION

Formation of the Cu(I)-GSH complexes, prepared two ways as described under "Experimental Procedures," was monitored by 'H NMR spectroscopy observing the broadening of the GSH resonances upon complexation (Fig. 1). The broadening depends on the decrease of the T₂ spin-lattice proton relaxation time, which is primarily due to the reduced mobility of GSH in the complex-bound form. From the NMR spectra, it is immediately obvious that the two complexes are identical. In spectrum b, the typical resonances of GSSG are also detectable after complex formation. Addition of Cu(II) causes oxidation of stoichiometric amounts of GSH to GSSG and complexation of the resulting Cu(I) with the remaining GSH.

At all GSH:copper ratios tested (2:1, 3:1, 5:1, 10:1, 20:1, and 50:1), GSH was able to form very stable complexes with Cu(I) even in the presence of oxygen. The 2:1 complex (0.7 mM copper) started to oxidize in air only after 5 h of incubation in a shaking water bath at 37 °C; the same behavior was observed with the 3:1 complex starting with GSH and Cu(II) regardless of whether it was formed in air or under nitrogen atmosphere. The reoxidation time was longer for complexes with higher GSH:Cu(I) ratios.

Fig. 2 shows the EPR spectrum of a 0.25 mM solution of Cu-free Zn-superoxide dismutase in 0.05 M phosphate buffer, pH 7.4, and that obtained immediately after the addition of
The peaks of the Cu(I) . GSH complex are marked with arrows. For the NMR conditions, see "Experimental Procedures."
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The reconstitution of Cu,Zn-superoxide dismutase from copper-free enzyme by Cu(I)-GSH in 0.05 M phosphate buffer, pH 7.4 (20% D$_2$O). The spectral region of the isotropically shifted resonances is reported. Spectrum a, 0.84 mM Cu-free, Co-superoxide dismutase; spectra b-d, same as spectrum a immediately after successive additions, in air, of the 1:3 Cu(I)-GSH complex (0.56 mM copper). For the NMR conditions, see “Experimental Procedures.”

With increasing Cu(I)-GSH (1:3) concentrations up to 1:1 (copper-protein monomer) molar ratio (spectra b-d), the spectrum changed until the final spectrum was similar to that of the Cu(I),Co enzyme as obtained by anaerobic reduction of the Cu(II),Co protein with borohydride in the presence of 20% D$_2$O (Fig. 6, spectrum a). However, two bands (A and F) were missing in the spectrum of the reconstituted enzyme (Fig. 5, spectrum d) with respect to the spectrum of Cu(I),Co-superoxide dismutase. On the other hand, addition of GSH to Cu(I),Co-superoxide dismutase produced a spectrum typical of the genuine protein. An explanation for the difference may be found in the fact that resonances A and F are due to mobile NH protons of cobalt-bound histidines (Bertini et al., 1985). Under the experimental conditions of Fig. 5 (spectrum d), these groups could be in a faster exchange with the solvent, resulting in the disappearance of their proton resonances. If this explanation is correct, the effect is likely to be related to a greater solvent accessibility and may be considered as a further, although indirect, indication for the presence of bulky ligand, such as Cu(I)-GSH, in the enzyme active site.

Reoxidation of the Cu(I)-GSH-protein complex restored the geometry of the coordination sphere of the native active site as monitored by paramagnetic NMR spectroscopy (Fig. 6). Also in this case, the process was accelerated by chromatography of the sample on Sephadex G-25.

Since full reconstitution of the protein was achieved in the experiments described above by adding copper as Cu(I)-GSH at a 1:1 ratio with respect to the available copper-binding sites of the protein, the relative reconstitution efficiency of Cu(II) complexes under comparable conditions was tested. In fact, Cu,Zn-superoxide dismutase is usually reconstituted from its apoprotein by adding inorganic cupric salts. The results obtained with CuSO$_4$, Cu(II)-GSSG, and Cu(II)-methionine are shown in Table I. It is evident that only Cu(I)-GSH was able to completely reconstitute the enzyme. Moreover, full reconstitution of the enzyme by Cu(I)-GSH occurred independ-ently of the GSH:copper ratio used between 2 and 500.

In conclusion, our results show that a very stable complex between copper and GSH is obtained even in the presence of oxygen and that this complex is able to donate Cu(I) to Cu-free,Zn-superoxide dismutase, most likely through the formation of a ternary complex, giving 100% reconstitution of the holoenzyme under conditions where Cu(I) cysteine is unstable and Cu(II) complexes are not able to fully reconsti-
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tute the enzyme. It is interesting to note that Cu(I)-GSH is able to reconstitute the copper-free enzyme even at high GSH:Cu(I) ratios, such as those existing within eukaryotic cells, where GSH is 10^{-3} M. On the basis of these results, intracellular GSH could be proposed as the best candidate for physiological copper donation to superoxide dismutase, in line with its role as copper chelator in copper-overloaded hepatoma cells (Freedman et al., 1989; Freedman and Peisach, 1989).

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