A FERROCYANIDE CHARGE-TRANSFER COMPLEX OF BOVINE SUPEROXIDE DISMUTASE. RELEVANCE OF THE ZINC IMIDAZOLATE BOND TO THE REDOX PROPERTIES OF THE ENZYME

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SUMMARY. Ferrocyanide does not reduce the bovine superoxide dismutase copper at pH 3.0 as it does at higher pH (1,2) but binds at the copper site giving a pink-violet charge-transfer complex. Similar reactions occur between ferrocyanide and Cu(II) bovine carbonic anhydrase or Cu(II) diethylenetriamine near neutral pH. The non-reducibility of superoxide dismutase Cu(II) at low pH suggests that its redox potential depends on the conformation of the site and on the presence of the zinc-imidazolate bond.

Bovine superoxide dismutase consists of two chemically identical subunits, each containing one Cu(II) and one Zn(II). The two metals are shown by X-ray data to be both bound to the imidazolate of His 61, which acts as a bridging group (3). Whilst Cu(II) is essential for activity, Zn(II) can be substituted for by a number of divalent cations as Co(II) (4,5), Hg(II), Cd(II), and Cu(II) (6) without loss of enzymatic activity.

In the pH range 5-8.7 superoxide dismutase copper is reduced by ferrocyanide (1,2). In this communication we report a study on the reactions of superoxide dismutase with ferrocyanide at lower pH, which may elucidate the protein reduction mechanism.

MATERIALS AND METHODS

Bovine superoxide dismutase was prepared as previously described (7). Bovine carbonic anhydrase was obtained as a byproduct in the preparation of superoxide dismutase (8). The Cu(II) enzyme was prepared by titration of the apoenzyme with $CuSO_4$ (8). Cu(II) diethylenetriamine was prepared by mixing equimolar amounts of $CuSO_4$

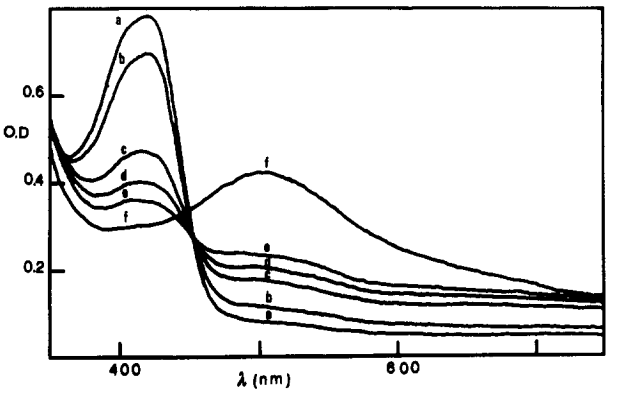

Fig. 1. Optical spectra of a ferrocyanide treated superoxide dismutase solution brought to acid pH. Curve a: unbuffered water solution of 0.4 mM superoxide dismutase, pH 5.5, plus 1.1 mM ferrocyanide; curve b: pH brought to about 3.5 with diluted HCl; curves c, d, e: same as b after 20, 70, 170 min. respectively; curve f: about one hour after the addition of more HCl, pH = 3.0. The temperature was of about 30 °C.

and diethylenetriamine in aqueous solution. Optical spectra were recorded on a DK-2A Ratio Recording Beckman Spectrophotometer.EPR spectra were obtained with a Varian V.4502 spectrometer.

RESULTS

Reaction of ferrocyanide with bovine superoxide dismutase

Fig. 1,a shows that excess ferrocyanide (1.3 equivs. per copper ion) added to an unbuffered protein solution (pH = 5.5) causes reduction of the copper (1,2) with bleaching of its absorption band at 680 nm and formation of free ferricyanide, monitored by the absorption band at 425 nm. Lowering the pH to pH 3.5 causes a slow increase of the absorbance above 455 nm, maximum at 500 nm, and a decrease of the 425 nm band (Fig. 1, b-e). On further acidification (pH = 3.0, Fig. 1,f) the 425 nm band disappears and the 500 nm band is fully formed (ε = 550 M^{-1} cm^{-1} per copper ion). The absorption of control ferricyanide solutions is not affected by acidification in the same pH range. In Fig. 2 the EPR spectra relevant to this experiment are reported. The isotropic spectrum of curve c, measured on the same solution of Fig. 1,f, shows the

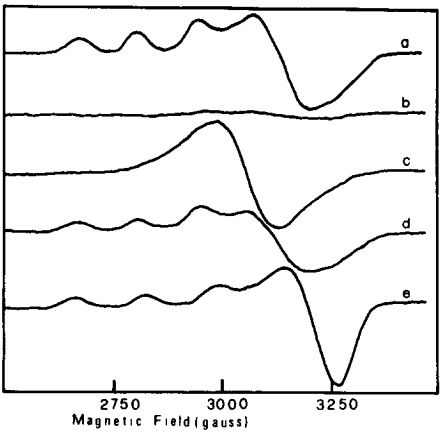

Fig. 2. EPR spectra of a ferrocyanide treated superoxide dismutase solution brought to acid pH. Curve a: unbuffered water solution of 0.4 mM superoxide dismutase pH 5.5; curve b: plus 1.1 mM ferrocyanide; curve c: same as b brought to pH 3.0 with diluted HCl (see Fig. 1 f); curve d: same as c dialyzed 24 hours against two changes of 0.1 M phosphate pH 7.4; curve e: same as a brought to pH 3.0 with diluted HCl. Acidification causes 10% dilution of the solutions of curves c - e. EPR conditions: temperature -170 °C; microwave frequency 9.15 GHz; microwave power 10 mw; modulation amplitude 10 gauss.

same saturation behaviour as the copper of "untreated" superoxide dismutase. It accounts for all the spin concentration present in the sample before the addition of ferrocyanide, in the error limits of double integration. Its apparent g value of 2.16 is very near to the average g value of the superoxide dismutase copper at pH 3.0 prior the addition of ferrocyanide (Fig.2,e). All these facts support the assignment of the isotropic spectrum to the copper of superoxide dismutase.

Addition of ferrocyanide to a previously acidified protein solution (pH 3.0-3.5) causes precipitation of a violet material. Dialysis against 0.1 M phosphate pH 7.4 of either the solution of Fig. 2,c or the solution containing the violet precipitate, gives a fairly good reversibility (Fig. 2,d).

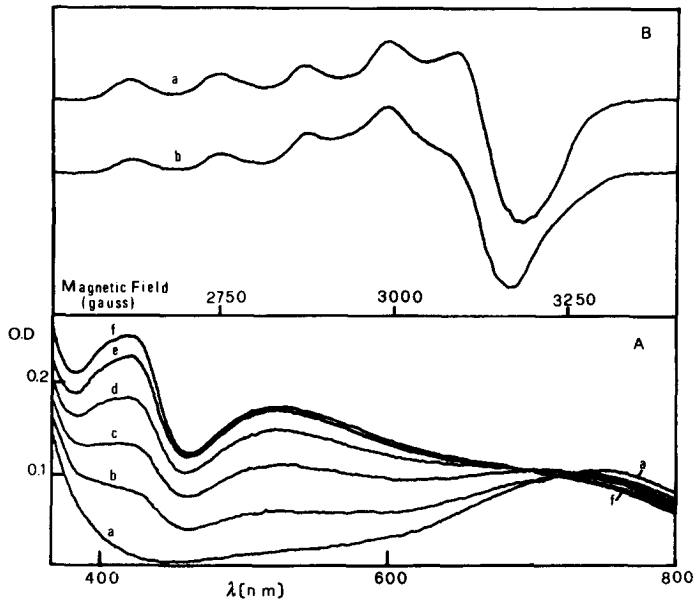

Fig. 3. Optical and EPR spectra of ferrocyanide treated bovine carbonic anhydrase. A: unbuffered water solution of 0.9 mM enzyme, pH 6.0 (a); plus 0.3, 0.6, 0.9, 1.2, 1.5 mM ferrocyanide respectively (b - f). B: 3.3 mM unbuffered aqueous enzyme, pH 6.0 (a); plus one equivalent ferrocyanide (b). EPR conditions as in Fig. 2.

Reaction with ferrocyanide of Cu(II) bovine carbonic anhydrase and Cu(II) diethylenetriamine.

Fig. 3,a shows the optical spectra of Cu(II) carbonic anhydrase reacted with increasing amounts of ferrocyanide in unbuffered aqueous solutions (pH $\simeq$ 6). Two new bands are formed at 425 and 525 nm. The copper band at about 730 nm decreases slightly and is shifted to shorter wavelength. A slight excess of ferrocyanide over one equivalent is required to fully develop the 525 nm band (ε = 200 $M^{-1}cm^{-1}$) whilst the 425 nm band undergoes further increases with either time or increasing ferrocyanide concentration. In phosphate buffered solutions at pH 6.0 competition is observed between ferrocyanide and the buffer anion, which is known to bind copper with an affinity constant of 6.7 x 10^3 M^{-1} (8). Alkalinization of a ferrocyanide treated solution abolishes the 525 nm band and only partly decreases the 425 nm band, which can then be assi-

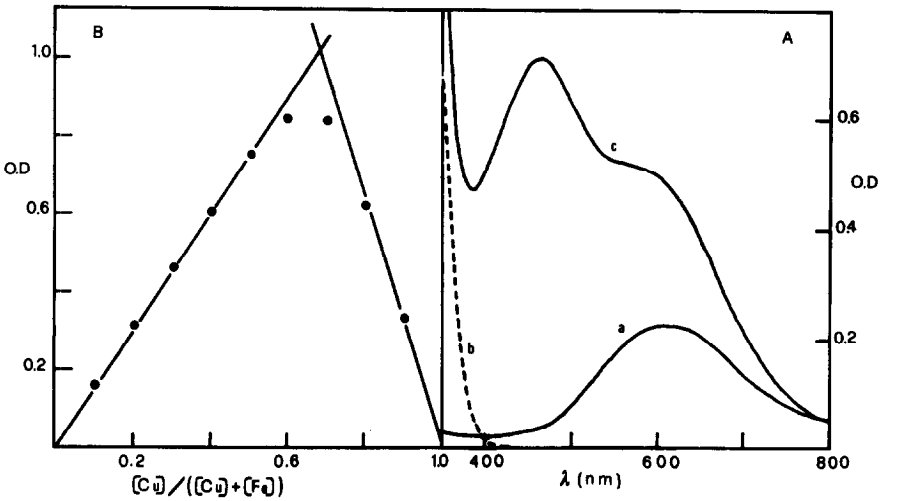

Fig. 4. A: optical spectrum of ferrocyanide treated Cu(II) diethylenetriamine. 2.5 mM Cu(II) diethylenetriamine (a); 5.0 mM ferrocyanide (b); 2.5 mM Cu(II) diethylenetriamine and 5.0 mM ferrocyanide (c). B: a Job's plot of the reaction of 5.0 mM Cu(II) diethylenetriamine with 5.0 mM ferrocyanide measured at 475 nm.

gned to free ferricyanide. The Fig. 3,b shows the EPR spectra of Cu(II) bovine carbonic anhydrase and of the same sample after addition of one equiv. of ferrocyanide. No further change occurs on addition of more ferrocyanide. Double integration shows a 15% decrease of signal intensity respect to the unreacted protein. The amount of ferricyanide formed in solution, as monitored by the 425 nm band, is in the range of 15% of Cu(II) initially present.

Fig. 4, A, shows the optical spectra of Cu(II) diethylenetriamine, of ferrocyanide and of a mixture of the two reactants. A new absorption is formed at 470 nm (ε = 300 M^{-1} cm^{-1}). The EPR spectrum of the copper complex is unaffected by ferrocyanide, indicating that no reduction of copper occurs. A Job's plot (9) shows that one molecule of ferrocyanide reacts with two molecules of the copper complex (Fig. 4, B).

DISCUSSION

The reaction of ferrocyanide with Cu(II) diethylenetriamine is very similar to that reported to occur with bis(L-histidinato) Co(III) (10), which is believed to produce a trinuclear adduct containing two Co(III) bridged by two cyanide anions to the same

Fe(II). This compound is characterized by an absorption band at 500 nm (ε = 480 M^{-1} cm^{-1} per Co) assigned to a Fe(II) $\longrightarrow$ Co(III) charge transfer. The corresponding absorption at 470 nm of the Cu(II) diethylenetriamine-ferrocyanide adduct can be assigned to an analogous charge transfer, since the EPR spectrum, unchanged after reaction, indicates that copper is still in the oxidized state. The similar energy of the charge transfer bands in the two cases probably reflects a similarity of redox potential of the two metal couples.

The reaction with ferrocyanide of Cu(II) carbonic anhydrase is very likely of the same type. The binding of ferrocyanide causes a distortion of the protein metal site recognizable from the rhombic shape of the EPR spectrum. This distorted species is more reducible than the axial Cu(II) diethylenetriamine derivative (11) as indicated by the 15% decrease of EPR signal intensity and by the shift of the charge transfer band to 525 nm. The intensity of this band is very low, probably due to a lesser orbital overlap in the strained molecule. It is also possible that the bridging cyanide is not directly bound to Cu(II) in the protein, but is hydrogen bonded to a water molecule of the Cu(II) inner coordination sphere as in the case of $Au(CN)_2^-$ bound to native Zn(II) human carbonic anhydrase C (12).

A similar Cu(II)-ferrocyanide adduct also appears to be formed between superoxide dismutase and ferrocyanide at pH $<$ 5. In Fig. 1 a species absorbing at 500 nm is formed by acidification of a ferrocyanide reacted superoxide dismutase solution. While this band is formed, the 425 nm band due to ferricyanide disappears. The increase of intensity in the 700 nm region, which is too remote to be entirely due to tailing of the 500 nm band and the EPR spectrum of Fig. 2,c, show that copper reoxidizes at low pH. The reason for the isotropy of the copper EPR signal is not yet clear.

A study of the superoxide dismutase-ferrocyanide system in the pH range 5-8.7 (2) suggested that a ligand with pK $\gg$ 9 is pro

tonated and released from the copper coordination sphere while reduction of copper occurs. This ligand is likely to be the imidazolate of the bridging His 61, as optical and EPR spectra of Co(II) superoxide dismutase are identical when copper is either reduced or removed (5,13). Recent data from this laboratory (14) have shown that low pH causes uncoupling of Cu(II) and Co(II) spins in Co(II) superoxide dismutase, upon breaking of the Co(II)-imidazolate bond and protonation of the Co(II) facing nitrogen of the imidazolate ring. In these conditions Cu(II) shows an axial EPR spectrum. The axial species is not reducible, as the axial Cu(II) diethylenetriamine (11). Thus the presence of the Zn(II)- or of the Co(II)-imidazolate bond appears to play an important role in determining the redox properties of the Cu(II). It probably helps the removal of the imidazolate from the pentacoordinated (3,11) copper, which gives the site a geometry more suited to Cu(I) (15). It is apparent that the chemical and steric requirements of a metal substituted for Zn(II) are not so crytical as are in copper substitution.

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