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MULTIPLE ELECTROPHORETIC VARIANTS OF Cu, Zn SUPEROXIDE DISMUTASE AS EXPRESSION OF THE ENZYME AGING. EFFECTS OF H_2O_2, ASCORBATE AND METAL IONS

Irene Mavelli[+], Maria Rosa Ciriolo[++] and Giuseppe Rotilio[++]

[+]Institute of Applied Biochemistry and CNR Center of Molecular Biology, University of Rome and [++]Department of Biology, IInd University of Rome, Rome, Italy

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SUMMARY. Multiple electrophoretic bands, with R_F identical to the natural molecular variants, are produced by treatment of purified Cu, Zn Superoxide dismutase with either H_2O_2 or ascorbate plus Fe(III) EDTA. The ascorbate reaction is also due to H_2O_2 since it is inhibited by catalase. However while H_2O_2 inactivates the enzyme, the electromorphs produced by ascorbate-Fe(III)-EDTA have only slightly less activity than the native enzyme and this property parallels the natural situation. It is concluded that oxidative aging can be responsible for the multiple molecular variants of the natural enzyme, under conditions where the oxidant attack is preferentially directed to amino acid side chains outside the active site. Such conditions may occur when a metal ion coordinated to the protein surface undergoes a redox cycle with biological reductants, like ascorbate.

Cu,Zn superoxide dismutase shows natural microheterogeneity, which produces a multiple pattern of at least three closely spaced bands in activity stained gel electrophoresis of most tissues (1). This heterogeneity persistsin highly purified enzyme preparations where at least two electrophoretic bands are usually detected by protein staining. In the case of the bovine enzyme, a careful investigation (2) was able to show that the three components which are detectable by activity staining of bovine hemolysates have different isoelectric points and identical antigenicity. Furthermore the two major forms, which have lower anodic mobility, were isolated and were found to have the same metal content, thus ruling out the hypothesis that they may correspond to partially metal-depleted molecules, which display comparable electrophoretic mobilities (3). They also have identical EPR spectra of the protein-bound copper and comparable activity which indicates substantial identity of the active site in the two molecular variants of the enzyme. The identical immunoreactivity of the electrophoretic forms (2) suggests that they are not genetically determined but may be related to some aging process of the enzyme. It should be recalled that Cu,Zn superoxide dismutases are very stable enzymes, however they are characteristically inac-

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tivated by H_2O_2 which is a product of superoxide dismutation (4, 5). Therefore, if the natural heterogeneity of Cu,Zn superoxide dismutase is due to some oxidative process, it can not be simply related to a reaction with H_2O_2 such as that already described in the isolated molecular system (4,5) as the natural variants have similar activity and identical EPR spectra and copper content (2). The aim of the present work was to consider other conditions that could produce an oxidant-induced alteration of the enzyme similar to that observed in the natural variants.

EXPERIMENTAL

Materials. Distilled water was thoroughly demetallized by exhaustive treatment with a metal-chelating resin (Chelex 100, Biorad). Bovine Cu,Zn superoxide dismutase was purified according to a standard method (6) and the copper-free protein was prepared as previously described (3). Ascorbate and ADP were purchased from Fluka, H_2O_2 and $FeCl_3$ from Merck, and catalase (bovine liver, doubly crystallized) from Sigma.

Methods. Superoxide dismutase activity was assayed polarographically (7) with an Amel polarographic unit, Model 465. Protein concentration was measured spectrophotometrically (8). Polyacrylamide gel electrophoresis was carried out as previously described (9). Densitometric analysis of gels was performed in an ISCO, Model 1310, gel scanner at 580 nm.

Incubation with ascorbate and H_2O_2. 0.3 mM Cu,Zn superoxide dismutase was incubated for 4 hours at 37°C with 10 mM ascorbic acid in 50 mM potassium phosphate buffer, pH 7.4 under gentle stirring in the absence and presence of iron, either 0.3 mM $FeCl_3$ or Fe(III) EDTA (0.3 mM $FeCl_3$ + 0.3 mM EDTA). The incubations were stopped by exhaustive dialysis against 50 mM potassium phosphate buffer at pH 7.4. Incubations in the presence of H_2O_2 were performed in similar conditions except that peroxide was added in 0.1 mM aliquots every 15 min., up to 1.4 mM. The reaction was stopped with 2 µM catalase.

RESULTS

As expected from previous data (4, 5) treatment of Cu,Zn bovine superoxide dismutase with H_2O_2 resulted in enzyme inactivation (Table I). A novel result, to our knowledge not yet reported, was that multiple electrophoretic

TABLE I

Oxidant-induced activity and electrophoretic changes of Cu,Zn bovine superoxide dismutase in relation to the natural electrophoretic variants

Conditions that give multiple electrophoretic bands	Activity %	Band multiplicity[2]				
- Untreated enzyme[1]	100	55 (0.26),	35 (0.22)	10 (0.34)		
- Treatment with H_2O_2	40	25 (0.26),	40 (0.29)	20 (0.33)	10 (0.36)	5 (0.40)
- Treatment with ascorbate plus Fe(III)EDTA	80	20 (0.26)	30 (0.30)	30 (0.34)	12 (0.38)	6 (0.41)

1) This situation refers to a fresh tissue, such as bovine hemolysate, analyzed by activity stained gel electrophoresis (2).

2) The first figure indicates the per cent intensity of the band as monitored by densitometric analysis of gels. The figure in brackets represents the band R_F.

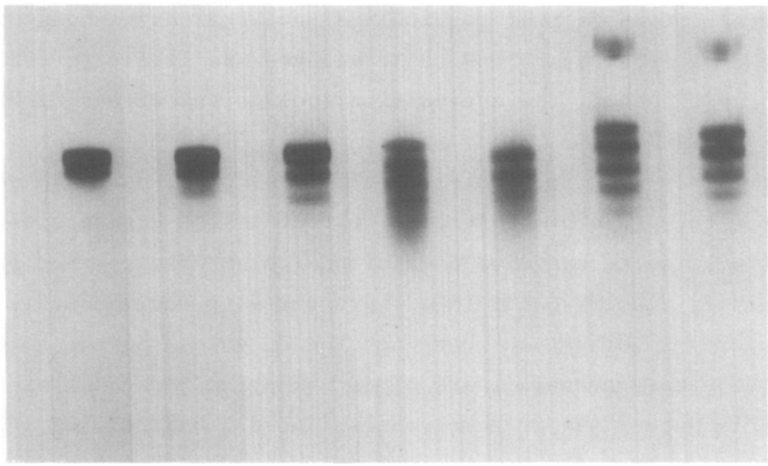

Fig. 1: Polyacrylamide gel electrophoresis of Cu,Zn bovine superoxide dismutase. From the left: the native protein; the same after treatment with ascorbate; the same after treatment with ascorbate plus $FeCl_3$; the same after treatment with ascorbate plus Fe(III)-EDTA; the Cu-free protein after treatment with ascorbate plus Fe(III)-EDTA; the native protein after treatment with H_2O_2; the same after treatment with H_2O_2 plus Fe(III). The slow band of the two last gels is catalase. For other conditions see text.

bands were apparent upon reaction with the peroxide (Fig. 1). Addition of Fe(III) salts was irrelevant to the magnitude of the effects observed. Moreover H_2O_2 was not able to change the electrophoretic pattern of the copper-free protein (not shown).

Incubation with ascorbate, in the presence of Fe(III)-EDTA, produced a manifold of electrophoretic bands with higher mobility than the major components of the native enzyme (Fig. 1). The band pattern was very similar to that obtained with H_2O_2; however the enzyme activity was decreased only slightly by the ascorbate-Fe(III) EDTA treatment (Table I). Moreover the presence of iron as the EDTA chelate was crucial for the electrophoretic effect to be observed to a high extent: ascorbate alone or in the presence of $FeCl_3$ had much slighter effect (Fig. 1). Similar modifications to those observed with the native protein were obtained by treating the copper-free protein with ascorbate-Fe(III) EDTA. All the effects induced by the ascorbate-Fe(III) EDTA treatment were abolished by catalase (not shown).

DISCUSSION

It is evident from the experiments reported here that H_2O_2 does in fact induce changes of the electrophoretic band pattern of bovine Cu,Zn superoxide dismutase, which are in the same direction as the natural heterogeneity. However, these changes were closely parallel to inactivation of the enzyme, and this is not the case of the natural molecular variants (2). Moreover the effects of H_2O_2 were found to be independent of the presence of iron and the copper-free protein was not affected by H_2O_2 treatment. It can be concluded

that addition of H_2O_2 does preferentially affect the enzyme-bound copper (4, 5) and that the parallel electrophoretic changes are likely to reflect destruction of residues at the active site such as the liganding histidines (4).

The experiments with ascorbate, on the other hand, point to a selective modification of the protein moiety with respect to the active site. In fact a large electrophoretic change of a magnitude comparable to that produced by H_2O_2 was observed, also in conditions where activity was minimally affected. It is particularly important to note that the R_F values of the three major bands obtained by this treatment are almost identical to those of the three variants usually detectable in hemolysates (Table I and ref. 2). It is also interesting to note that the slight activity decrease observed in the presence of ascorbate-Fe(III) EDTA, which drastically increases the relative amount of more mobile bands (Table I), is comparable to the lower activity of the isolated more mobile band of the two major natural variants (2).

The effect of ascorbate in the presence of Fe(III) EDTA was abolished by catalase. It is therefore an H_2O_2 effect, although apparently directed to amino acid residues outside the active site. In line with this interpretation ascorbate plus Fe(III) EDTA was able to induce a comparable modification of the electrophoretic pattern of the copper-free protein as well, contrary to what observed with H_2O_2.

Thus relatively large changes of electrophoretic mobility of Cu,Zn superoxide dismutase are not necessarily accompanied by metal loss or damage of the active site residues. In fact the activity of the ascorbate-treated samples resulted to be the same, whether calculated on a metal or on a protein basis. The role of Fe(III) EDTA in producing this effect on the protein moiety of Cu,Zn superoxide dismutase in the presence of ascorbate can be explained by a peculiar efficiency of this chelate in catalysing the autoxidation of ascorbate. Alternatively, Fe(III) EDTA could coordinate the amino acid side chains to which the H_2O_2 effect is then selectively directed. The H_2O_2 produced by the autoxidation of ascorbate can also reoxidize the ascorbate-reduced iron in a Fenton type reaction to give strongly oxidizing OH radicals. OH radicals are highly reactive species which react preferentially with molecules surrounding the site where they are produced. This is the mechanism by which H_2O_2 destroys the copper-liganding histidines of Cu, Zn superoxide dismutase (4,5). A similar mechanism may also apply to amino acid side chains outside the active site if specific coordination sites for Fe(III) EDTA were available on the protein surface. It is still unknown which amino acid side chains are involved in the reaction with ascorbate Fe(III) EDTA. This point, in relation to the chemical nature of the diversity of the natural electrophoretic forms of the enzyme, is under study in this laboratory.

A final comment should be made concerning the fact that ascorbate and iron are indeed likely to be involved also in the in vivo production of superoxide dismutase heterogeneity. Dehydroascorbate is known to penetrate the cell membrane and once within the cells it is reduced to ascorbate (10). Iron is available in large amounts from heme degradation and, in red blood cell precursors, from transferrin. Reduction of iron by ascorbate has been involved in both iron uptake by cells (11) and iron release from intracellular deposits (12).

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