ISOLATION OF A NEW COPPER - CONTAINING SUPEROXIDE DISMUTASE

BACTERIOCUPREIN

K. Puget and A. M. Michelson

Institut de Biologie Physico-Chimique
13, rue P. et M. Curie - 75005 Paris.

Received April 25,1974

SUMMARY. - A copper and zinc containing superoxide dismutase has been isolated from marine bacteria. Purification and characteristics of this enzyme are described and the evolutionary implications of the presence in procaryotes of this kind of superoxide dismutase (formerly characteristic of eucaryotes) are discussed. Bacteriocuprein differs from erythrocuprein in that the enzyme contains one atom of copper (and not two) and two atoms of zinc, as well as in the ultraviolet absorption spectrum.

In numerous publications by Fridovich and his collaborators (1 - 3) it has been postulated that the molecular properties of superoxide dismutase (SOD) have been rigidly preserved during evolution of eucaryotes and that all eucaryotes should contain SOD with properties similar to bovine erythrocupreine, whereas all procaryotes should contain the distinct manganese containing enzyme characteristic of Escherichia coli (4). Indeed such a hypothesis has been cited in discussions (5) on the origins of organelles of eucaryotes cells, particularly since mitochondrial SOD in an as yet limited number of species (6) is a manganese enzyme similar to that isolated from E. coli.

We have already shown that the major superoxide dismutase from the eucaryote mushroom Pleurotus olearius is a manganese enzyme (7) containing two atoms of manganese but with a molecular weight twice that of the enzyme from E. coli. The properties of this enzyme suggested that at least in certain branches of aerobic evolution, the SODs of certain eucaryotes and procaryotes have a common origin, in contradiction to the hypothesis of Fridovich and his collaborators that the two classes of SODs could not be derived from a common ancestral protein but reflect rather independent lines of evolution of these two classes of cells after passage to aerobic life (2, 3).

We have also shown that the major SOD in certain marine bacteria (Photobacterium leiognathi and Photobacterium sepia and about 20 other strains of symbiotic and free living bioluminescent species) is in fact an iron containing enzyme and that two immunochemically distinct kinds of iron enzymes exist (8). We now, by the isolation of a copper and zinc containing SOD, bacteriocuprein, from bacteria, (Photobacterium leiognathi) present evidence suggesting that the original hypothesis of Fridovich of functional convergence from polyphyletic origins, must be abandoned and that in fact with respect to SOD activities there may well have been a monophyletic evolutionary process from procaryote to eucaryote, which began at the moment of transition from anaerobic to aerobic life conditions.

METHODS

Assay of enzymatic activity. The activity of superoxide dismutase was measured by inhibition of the chemiluminescent reaction produced in the system : oxygen - hypoxanthine - xanthine oxidase - luminol. Superoxide dismutase in competition with luminol for superoxide anions generated by the xanthine oxidase system decreases light emission (8, 9).
Photobacterium leiognathi were grown as described elsewhere (8).

RESULTS

Purification of bacteriocuprein from Photobacterium leiognathi. Bacteria (2 kilo, wet weight) were lysed by stirring in cold distilled water (8 litres) for 3 hours at 4° and then centrifuged at 16 300 g for 10 min at 4°. The residues were dispersed (Waring blendor) in 9 l of water and left at 4° overnight, then centrifuged as before. To the clear combined supernatants was added KCl to a final concentration of 0.1 M (final pH 6.5) and the solution was then heated at 58° C for 3 min by circulation through a coil at this temperature. The resultant suspension of proteins was cooled to 4° and clarified by centrifugation at 16 300 g for 5 min at 4°. The supernatant was concentrated to 6 l by ultrafiltration using a fiber filter HIDP10 and then brought to 25 % saturation with ammonium sulphate by dialysis against 2 l of saturated ammonium sulphate. Precipitated material was removed by centrifugation and discarded. The active fraction of superoxide dismutase was precipitated from the supernatant by addition of ammonium

sulphate to 75 % saturation and this precipitate was dissolved in 5 x 10^{-2} M glycine buffer pH 8. 6 containing 5 x 10^{-2} M NaCl, and dialysed overnight against the same buffer. The solution (1 litre) was then applied to a column of DEAE-Sephadex A-50 (5. 4 x 54 cm) equilibrated with 5 x 10^{-2} M glycine, 5 x 10^{-2} M NaCl, pH 8. 6. Protein was eluted from the column with a linear gradient of 5 x 10^{-2} M glycine, 5 x 10^{-2} M NaCl to 3 x 10^{-1} M glycine, 5 x 10^{-1} M NaCl, pH 8. 6. The first active fraction of superoxide dismutase (5. 7 g, 10. 9 x 10^{6} units) was not retained by the column and appeared in the volume obtained on washing with 5 x 10^{-2} M glycine, 5 x 10^{-2} M NaCl pH 8. 6. The second superoxide dismutase was eluted at 2. 0 x 10^{-1} glycine 3. 5 x 10^{-1} NaCl, pH 8. 6 and was the non-haematinic ferroprotein described elsewhere (8).

Fractions containing the first superoxide dismutase activity were dialysed against 5 x 10^{-4}M phosphate buffer, pH 7. 4, filtered through a column of DEAE-cellulose (3. 3 x 40 cms) in the same buffer, and then adsorbed on to a column of carboxymethyl cellulose (CM - 52, 3. 5 x 32 cm). A linear gradient of phosphate (750 ml each of 5 x 10^{-4} and 10^{-1} M) pH 7. 0 was applied. Superoxide dismutase (0. 23 g, 6. 7 x 10^{6} units) was eluted at a phosphate concentration of 2. 6 x 10^{-2} M. The purified dismutase was concentrated and stored as a precipitate in saturated ammonium sulphate solution, or maintained frozen at - 20°.

Molecular weight. Measurements of sedimentation velocity were made with a Beckman Spinco model L 2 - 65 B ultracentrifuge according to the method of Martin and Ames (10) with a linear 5 to 20 % (W/V) sucrose gradient. An SW 65 K rotor was used and centrifugation was for 17 hours at 45 000 rpm at 3° with suitable markers. The sedimentation coefficient for superoxide dismutase was 2. 7 using as references the following proteins : yeast alcohol dehydrogenase (7. 4), horse liver alcohol dehydrogenase (4. 82), and cytochrome C (1. 45). The molecular weight of native dismutase was calculated to be 33, 070.

Acrylamide gel electrophoresis. At the final stage of purification the dismutase gave a single band of protein (and activity) on gel electrophoresis carried out according to the procedure of Davis (11). Electrophoresis was performed in 7. 5 % acrylamide gel with Tris-glycine buffer, pH 8. 5 and

a constant current of 4 mA per gel. The protein was stained using Coomassie blue in 7 % acetic acid and destained with 7 % acetic acid.

Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis was performed according to Weber and Osborn (12). The samples were incubated at 37° for 2 hours in 10^{-2} M phosphate buffer, pH 7.0, 1 % in SDS, and 1 % β - mercaptoethanol, in the presence of 8 M urea ; 10 % acrylamide solutions were used for the preparation of the gels and electrophoresis was carried out for 2 hrs at a constant current of 16 mA per gel. Bacteriocuprein gave two bands with Rf values of 0.75 and 0.79 as compared with those obtained for yeast alcohol dehydrogenase 0.41, myoglobin 0.72, lysozyme 0.83 and cytochrome c 0.91. The native enzyme therefore consists of two different subunits of molecular weight 17,000 and 15,500.

Isoelectric point. The isoelectric point was determined on a LKB 8101 electrofocusing column with Ampholine (pH 7 to 9) in a sucrose gradient. Bacteriocuprein showed an isoelectric point of 8.25.

Optimum of pH. The pH dependance of dismutase activity determined either with xanthine oxidase/hypoxanthine and luminol, or by inhibition of reduction of cytochrome c (14) showed a maximum at pH 9.

Stability of superoxide dismutase activity. Bacteriocuprein showed high thermal stability ; no changes in enzymatic activity were found after incubation for 60 min at 20°, 30°, 40° or 50° C at pH 7.8. Incubation for 120 min at 60° caused a 58 % decrease in the activity whereas the enzyme was completely inactivated after 90 min at 70° C (Fig. 1). The stability of dismutase activity as a function of pH was determined after a 30 min preincubation at 20° in buffers ranging from pH 4 to 11. Activity was then studied by the standard test at pH 9.0. Bacteriocuprein was completely stable between pH 5 and 11 but at pH 4.0 lost 65 % of the original activity.

Metal determinations. Quantitative estimations of copper, zinc and iron were made by atomic absorption analysis using an Instrumentation Laboratories IL 253 Spectrometer. One atom of copper (1.02) and two atoms of zinc (2.5) per molecule of superoxide dismutase were found. Neither manganese or iron could be detected. Calculations were based on protein concentrations determined by M. Golberg (Institut Pasteur, Paris) by the method of fringes using the interference optics of a Spinco Model E ultracentrifuge.

The ultraviolet absorption spectrum using a Cary 14 is given in fig. 2. A

TABLE I

Effect of oxygen on superoxide dismutase activities

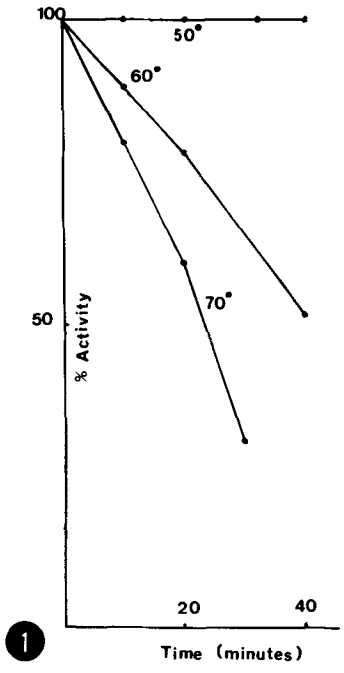
	Units		Ratio of units O_2/air
	Air	Oxygen	
Total units	28 220	102 480	3.63
Bacteriocuprein	12 920	28 880	2.23
Ferro SOD	15 300	73 600	4.81
Ratio units of Ferro SOD/cupro SOD	1.18	2.55	

solution of 1 mg bacteriocuprein/ml has an absorption of 0.651 at 280 nm and 0.434 at 260 nm. The spectra of human and of bovine erythrocuprein are also shown for comparison. Solutions at 1 mg/ml of these enzymes show an absorption of 0.58 at 265 nm (human) and 0.3 at 258 nm (bovine). In the visible region bacteriocuprein has λ max at 690 nm (670 - 710 nm) with ε max approx. 100 in accord with a single atom of copper per molecule of enzyme. (We thank Dr. R. C. Bray for this determination).

Effect of partial pressure of oxygen. Samples (5 gm wet weight) of Photobacterium leiognathi were grown in liquid media (8) with either air or pure oxygen. Each was then dispersed in water (20 ml) and sonicated for 1 min and then centrifuged. The debris was resuspended in 20 ml of 5×10^{-4} M phosphate pH 7.4 and resonicated for 2 min and again centrifuged. The combined supernatants were adjusted to 0.1 M KCl, heated at 55° for 3 min then cooled and centrifuged. Fractionation with ammonium sulphate (33 - 80 % saturation cut) by dialysis against appropriate quantities of saturated ammonium sulphate gave crude superoxide dismutase which was separated into bacteriocuprein and the ferroprotein SOD on a small column of DEAE-sephadex A-50 using a gradient of 5×10^{-2} M glycine 5×10^{-2} M NaCl pH 8.6 to 0.3 M glycine 0.5 M NaCl pH 8.6 (Table I).

DISCUSSION

The significance of a bacterial copper and zinc containing SOD



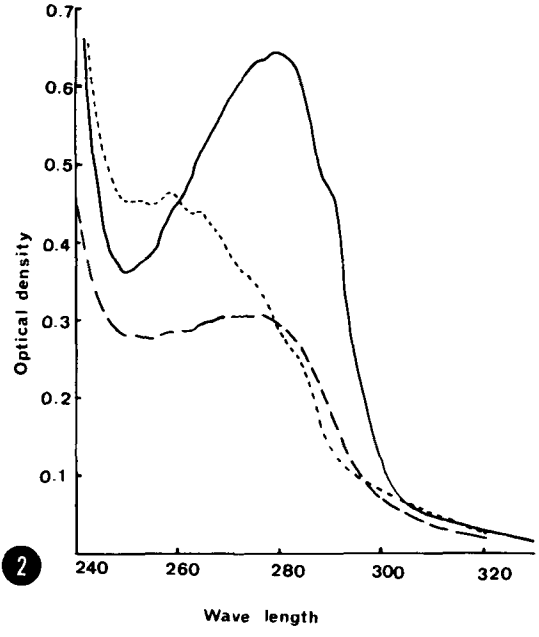


Fig. 1 Thermal stability of bacteriocuprein. Solution of 78 μg protein/ml in 5 x 10^{-3} M phosphate, pH 7.8.

Fig. 2 Ultraviolet absorption spectra of bacteriocuprein (——), of bovine erythrocuprein (---), and of human erythrocuprein (– – –) in 5 x 10^{-3} M phosphate pH 7.4.

with respect to evolutionary hypotheses of eucaryote cells has been discussed above. Of perhaps equal significance is the absence of a mangano-enzyme in these bacteria - no trace could be detected in Photobacterium leiognathi, though this does not exclude the possible presence of a mangano SOD in other marine bacteria.

The effect of increased partial pressure of oxygen (approximately 5 fold) is interesting in that preferential synthesis of the ferro enzyme is observed though some stimulation of bacteriocuprein also occurs (Table I). This is in contrast with Escherichia coli which also contains a ferro superoxide dismutase (different from that of Photobacterium leiognathi) with a second enzyme containing manganese. In this case the iron enzyme (although periplasmic) apparently does not vary in response to changes in p O_2 during

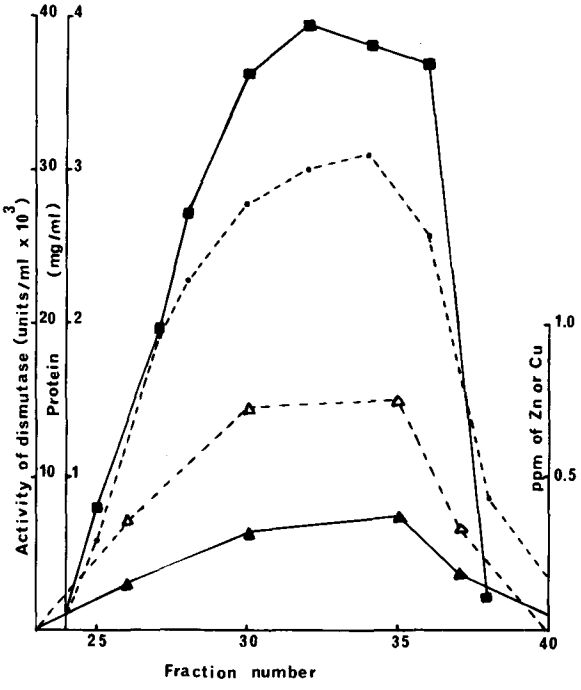


Fig. 3 Chromatography of bacteriocuprein on DEAE-cellulose using a phosphate (pH 7.4) gradient between 5 x 10^{-4} and 0.1 M. Protein, in mg/ml -●-●-, SOD activity (units/ml)-■-■- , zinc (μg/ml) -Δ-Δ- and copper (μg/ml) -▲-▲-.

growth whereas the level of the mangano enzyme is markedly dependent and shows a strong increase with increase in p O_2 (15, 16).

The ultraviolet absorption spectrum of bacteriocuprein is quite different from that of bovine erythrocuprein (λ max 258 nm) which contains no tryptophane. Some similarity with the absorption spectrum of human erythrocuprein can be seen. The presence of both copper and zinc (and the 1 : 2 ratio of the two metals) in bacteriocuprein is clearly shown in Fig. 3 in which it can be seen that both metals follow the activity of the enzyme. Other metals (such as iron) present only in traces, showed no correlation with enzyme activity. Incubation of bacteriocuprein with 10^{-5} M copper sulphate overnight at 4° C followed by dialysis, had no effect on the specific activity of the enzyme. It is therefore unlikely that a labile copper atom was "lost" from the enzyme during purification.

A number of differences can be seen between bacteriocuprein and erythrocuprein (bovine or human), apart from the number of atoms of

copper per molecule. Thus the specific activity of the bacterial enzyme is considerably less than that of bovine erythrocuprein (approximately half) in accord with the presence of a single copper centre. Whereas identical sub units are present in the mammalian enzymes, bacteriocuprein has two different sub units. In addition, the bacterial superoxide dismutase is a much more basic protein with an isoelectric point 8.25 compared with 4.95 for bovine (17) and 4.6 for human erythrocuprein (18), and is readily separated from the erythrocupreins by electrophoresis on acrylamide gels (relative R_{fs}, 0.13 for bacteriocuprein, 0.25 for bovine erythrocuprein and 0.36 for the human enzyme). No immunological cross reactions could be detected among the three superoxide dismutases, despite the very close similarity of molecular weights and the presence of copper and zinc in all cases. The thermal and pH stability of bacteriocuprein is striking, and in this respect resembles the two erythrocupreins. Finally, the inhibitory effect of cyanide is similar to that reported (19) for human erythrocuprein (50 % inhibition at 1.6×10^{-5} M KCN with 2×10^{-6} M enzyme) and is 25 % at 10^{-6} M KCN, 50 % at 2.2×10^{-6} M and 75 % at 4.8×10^{-6} M KCN for 2×10^{-11} M bacteriocuprein at pH 9.

The presence of a single copper site implies alternate reduction and oxidation of the metal in the mechanism of dismutation and zinc is presumably present for structural stability. Bacteriocuprein has been crystallized by dialysis against ammonium sulphate solutions.

REFERENCES

1. Mc Cord, J. M., Keele, B. B., and Fridovich, I. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1024-1027.
2. Goscin, S. A., and Fridovich, I. (1972) Biochim. Biophys. Acta 289, 276-283.
3. Misra, H. P., and Fridovich, I. (1972) J. Biol. Chem. 247, 3410-3414.
4. Keele, B. B., Mc Cord, J. M., and Fridovich, I. (1970) J. Biol. Chem. 245, 6176-6181.
5. Cohen, S. S. (1973) American Scientist 61, 437-445.
6. Weisiger, R. A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.
7. Lavelle, F., Durosay, P., and Michelson, A. M. (1974) Biochimie, in press.
8. Puget, K., and Michelson, A. M. (1974) Biochimie, in press.
9. Lavelle, F., Michelson, A. M., and Dimitrijevic, L. (1973) Biochem. Biophys. Research Communs 55, 350-357.
10. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372-1379.

11. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
12. Weber, K., and Osborn, M. J. (1969) J. Biol. Chem. 244, 4406-4412.
13. Warburg, O., and Christian, W. (1941/1942) Biochem. Z. 310, 384-421.
14. Lavelle, F., Puget, K., and Michelson, A. M., in preparation.
15. Gregory, E. M., and Fridovich, I. (1973) J. Bacteriol. 114, 1193-1197.
16. Gregory, E. M., Yost, F. J., and Fridovich, I. (1973) J. Bacteriol. 115, 987-991.
17. Bannister, J., Bannister, W., and Wood, E. (1971) Eur. J. Biochem. 18, 178-186.
18. Stansell, M. J., and Deutsch, H. F. (1965) J. Biol. Chem. 240, 4306-4311.
19. Bannister, J. V., Bannister, W. H., Bray, R. C., Fielden, E. M., Roberts, P. B., and Rotilio, G. (1973) FEBS Letters 32, 303-306.