Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 89-94

IN VITRO SYNTHESIS OF SUPEROXIDE DISMUTASES OF RAT LIVER

K. Hirano, M. Fukuta, T. Adachi, K. Hayashi,
M. Sugiura, Y. Mori and K. Toyoshi

Gifu Pharmaceutical University, 6-1, Mitahora-higashi 5-chome,
Gifu 502, Japan

Received April 18, 1985

SUMMARY: The syntheses of copper, zinc-superoxide dismutase (Cu, Zn-SOD) and manganese-superoxide dismutase (Mn-SOD) in vitro were studied. Both Cu,Zn-SOD and Mn-SOD were preferentially synthesized by free polysomes. Mn-SOD was synthesized as a large precursor (26,000 daltons), which was processed to the mature size (22,500 daltons) by in vitro incubation with a rat liver mitochondrial fraction. On the other hand, Cu,Zn-SOD was synthesized as the mature size product. It was shown that Cu,Zn-SOD and Mn-SOD synthesized in vitro represented 0.018% and 0.016% of the total translation products of free polysomes, respectively.

INTRODUCTION: Superoxide dismutase (EC. 1.15.1.1, SOD) is the enzyme that catalytically scavenges superoxide anion radicals and thus provides a defense against oxygen toxicity (1). Eukaryotic cells contain two distinct forms of SODs, copper, zinc-containing enzyme (Cu,Zn-SOD) in the cytoplasm and manganese-containing enzyme (Mn-SOD) in the mitochondria (2). The locus for human Cu,Zn-SOD was assigned to human chromosome 21 (3). A strain of a petite mutant of Saccharomyces cerevisiae which lacks mitochondrial DNA was found to contain Mn-SOD (2). This indicates that Mn-SOD is probably synthesized in the cytoplasm and then somehow transported to the mitochondria. Evidences have accumulated that the syntheses of SODs are induced in both prokaryotes and eukaryotes by agents which increase the steady state concentration of superoxide anion radicals (4, 5) and by

Abbreviation used: SOD, superoxide dismutase [superoxide: superoxide oxidoreductase (EC 1.15.1.1.)].

0006-291X/85 $1.50

prolonged hyperoxic exposure (6-8). On the other hand, the regulation of the intracellular contents of SODs has not been reported.

Therefore, in this work we attempted to synthesize SODs *in vitro* to clarify the mechanism of intracellular transport of SODs. The data reported here show that Cu,Zn-SOD of rat liver is synthesized as the mature size product and Mn-SOD is initially synthesized as a larger precursor, which is then converted to the mature form by the rat mitochondrial fraction.

MATERIALS AND METHODS

Purification of SODs from Rat Liver and Preparation of Rabbit Anti-SODs Antibodies : Cu,Zn-SOD was purified according to the method of Weisiger and Fridovich (9) and Mn-SOD according to the method of Salin et al. (10). Each purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Each antibody was prepared by immunizing a rabbit with each purified enzyme. The specificity of the antibodies was determined by immunoelectrophoresis.

In Vitro Protein Synthesis: Free and bound polysomes from rat liver were isolated by the method of Blobel and Potter (11). *In vitro* protein synthesis was carried out in a nuclease-treated rabbit reticulocyte lysate system (12) with 200 μCi/ml of [^{35}S] methionine (1143 Ci/mmol, New England Nuclear, NEN), 20 A_{260}units/ml of polysomes and 150 μM of each protease inhibitor (leupeptin, chymostatin, antipain and pepstatin). The reaction mixtures (60 μl) were incubated at 25°C for 90 min.

Trichloroacetic acid-insoluble radioactivity was measured according to the method of Roberts and Paterson (13).

Immunoprecipitation and Analysis of Translation Products: Immunoprecipitation was carried out with 50 μg of either anti-Cu,Zn-SOD or anti-Mn-SOD antibodies by the method of Mori et al. (14) with some modification; protein A-coated *Staphylococcus* cells (Sigma Chemical Co.) were added to the reaction mixture after *in vitro* protein synthesis before the reaction with each antibody. SDS-PAGE was carried out by the method of Laemmli (15) using 15% and 12.5% polyacrylamide gels for analysis of Cu,Zn-SOD and Mn-SOD, respectively. The gels were then treated with EN^{3}HANCE (NEN), dried and fluorographed (16).

The *in vitro* synthesized SOD contents were determined by the following method. After electrophoresis, the gels were cut into 1 mm slices, and slices were dissolved in toluene scintillant containing 3% PROTOSOL (NEN), 0.4% OMNIFLUOR (NEN), and then the radioactivity was counted.

Incubation of in Vitro Products with Subcellular Fractions: Subcellular fractionation of rat liver was performed as described by Takesue and Sato (17). *In vitro* protein synthesis was performed under the above conditions without protease inhibitors. The reaction mixtures were incubated at 37°C for 90 min with nuclear, heavy mitochondrial, light mitochondrial, microsomal and cytosolic fractions. The mixtures were analyzed as described above.

RESULTS AND DISCUSSION

Free or bound polysomes of rat liver were translated in a mRNA dependent rabbit reticulocyte lysate system and the products were analyzed by immunoprecipitation and SDS-PAGE as described in MATERIALS AND METHODS. Figure 1 shows a typical fluorogram of *in vitro* products.

The translation products that reacted with each of the antibodies, anti-Mn-SOD and anti-Cu,Zn-SOD, appeared in different regions (lanes 2 and 3). To confirm that the translation products that reacted with the corresponding antibodies are in fact subunits of newly synthesized Mn-SOD and Cu,Zn-SOD products, we examined their ability to compete with purified Mn-SOD and

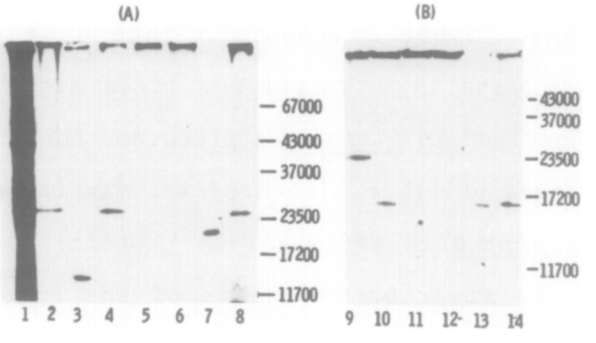

Figure 1. SDS-polyacrylamide gel electrophoresis of the translation products.
Free (lanes 1-5, 8, 9-11 and 14) or bound (lanes 6 and 12) polysomes of rat liver were translated using a nuclease-treated reticulocyte lysate system, and the translation products were analyzed after immunoprecipitation from about 2.2 X 10^6 d.p.m. of free polysomes or 2.5 X 10^6 d.p.m. of bound polysomes by SDS-PAGE, with detection by fluorography as described MATERIALS AND METHODS. (A) and (B) show the patterns on 12.5% and 15% polyacrylamide gels, respectively.
The translation products were recovered by adding (lane 2) or not adding (lane 1) protein A-coated cells to the reaction mixture before immunoprecipitation with anti-Mn-SOD antibody. Immunoprecipitation was performed with anti-Mn-SOD antibody (lanes 4, 6, 8 and 9) or with anti-Cu,Zn-SOD antibody (lanes 3, 10, 11 and 14). Lanes 5 and 11 are the same as lanes 4 and 10 except that the immunoprecipitation was performed in the presence of an excess of 50 μg of purified Mn-SOD and 100 μg of purified Cu,Zn-SOD, respectively. Lanes 7 and 13 are purified Mn-SOD and Cu,Zn-SOD, respectively, stained with Coomassie Brilliant Blue R-250.
Markers: bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 43,000), alcohol dehydrogenase (M.W. 37,000), γ-globulin L chain (M.W. 23,500), myoglobin (M.W. 17,200) and cytochrome c (M.W. 11,700).

Cu,Zn-SOD for the corresponding antibodies. The appearance of the translation products bands (lanes 4 and 10) was suppressed competitively by the presence of an excess of the corresponding proteins (lanes 5 and 11).

When bound polysomes were translated, translation products that reacted with each of the antibodies were detected (lanes 6 and 12). These results indicate that free polysomes are the preferential site of synthesis of both enzymes. We determined the molecular weights of the subunits of the Mn-SOD and Cu,Zn-SOD products. The apparent molecular weight of newly synthesized Mn-SOD (lane 8) was about 3,500 daltons larger than that of the mature subunit (lane 7, 22,500 daltons). On the other hand, the synthesized Cu,Zn-SOD (lane 14) had an identical subunit molecular weight (15,500 daltons) to that of the subunit of mature Cu,Zn-SOD (lane 13). Therefore, it is highly likely that the Mn-SOD synthesized *in vitro* is a precursor form of mature Mn-SOD as in the cases of other mitochondrial matrix proteins (18).

We also attempted to estimate the proportions of Cu,Zn-SOD and Mn-SOD in rat liver on the basis of the incorporation of [^{35}S]methionine into protein. Total translation products were determined by TCA precipitation, and the SOD products counted by the methods described in MATERIALS AND METHODS. It was found that the Cu,Zn-SOD and Mn-SOD products correspond to 0.018% and 0.016% of the total translation products of free polysomes, respectively. The amino acid compositions of Cu,Zn-SOD and Mn-SOD of rat liver were reported (7, 10). Considering the proportions of SOD activities in rat liver (19), it is assumed that [^{35}S] methionine radioactivity of the Cu,Zn-SOD products is three-fold higher than that of Mn-SOD products in this system with free polysomes. But we did not find a large difference in the proportions of SODs synthesized *in vitro*. We assumed that

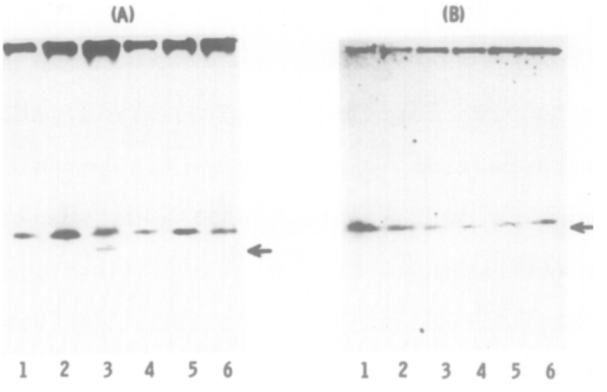

Figure 2. Conversion of SODs synthesized in vitro by subcellular fractions.
Sixty µl of the translation mixtures were incubated with 40 µl of buffer alone (20 mM Tris-HCl, pH 7.45, containing 0.25 M sucrose) or with the indicated amounts of subcellular fractions suspended in this buffer, followed by analysis after immunoprecipitation by SDS-PAGE as described in MATERIALS AND METHODS. Lane 1: buffer alone; lane 2: 380 µg of nuclear protein; lane 3: 420 µg of heavy mitochondrial protein; lane 4: 400 µg of light mitochondrial protein; lane 5: 450 µg of microsomal protein; lane 6: 340 µg of cytosolic protein.
(A): 12.5% polyacrylamide gel, immunoprecipitation with anti-Mn-SOD antibody;
(B): 15% polyacrylamide gel, immunoprecipitation with anti-Cu,Zn-SOD antibody.
The arrows in (A) and (B) indicate the positions of purified Mn-SOD and Cu,Zn-SOD, respectively.

this inconsistency is due to from a difference in intracellular metabolism between the two enzymes or the possibility that in Mn-SOD products, some methionines are present in the 3,500 daltons peptide.

As shown in Figure 2, when the products synthesized in vitro were incubated with the heavy mitochondrial fraction, the radioactivity appeared in the region corresponding to the mature Mn-SOD subunit as well as in the precursor region on a polyacrylamide gel. After incubation with the other fractions, however, no conversion of the 26,000 daltons product to the 22,500 daltons mature form was observed. On the other hand, when Cu,Zn-SOD products were incubated with all the fractions, no change in the position of the corresponding bands were detected.

These experiments suggest that Mn-SOD is synthesized on cytoplasmic free polysomes as the precursor and then imported

post-translationally into the mitochondria. Human Cu,Zn-SOD synthesized *in vitro* by fibroblast mRNA had the same molecular weight as the *in vivo* counterpart (20). This indicates that an N-terminal extra peptide is not present in human Cu,Zn-SOD. The nucleotide sequence of human Cu,Zn-SOD mRNA was elucidated, and it had an initial methionine as an extra amino acid (21). In our experiments, a Cu,Zn-SOD precursor in rat liver was not detected.

REFERENCES

1. Fridovich, I. (1975) Ann. Rev. Biochem. **44**, 147-159.
2. Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. **248**, 4793-4796.
3. Smith, G.F., and Burg, J.M. (1976) Down's Anomaly, Churchill Livingston, Edinburgh, pp. 234-251.
4. Hassan, H.M., and Fridovich, I. (1977) J. Biol. Chem. **252**, 7667-7672.
5. Dryer, S.E., Robert, L.D., and Autor, A.P. (1980) J. Biol. Chem. **255**, 1054-1057.
6. Hassan, H.M., and Fridovich, I. (1977) J. Bacteriol. **129**, 1574-1583.
7. Crapo, J.D., and McCord, J.M. (1976) Am. J. Physiol. **231**, 1196-1203.
8. Stevens, J.B., and Autor, A.P. (1977) J. Biol. Chem. **252**, 3509-3514.
9. Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. **248**, 3582-3592.
10. Salin, M.L., Day, E.D., Jr., and Crapo, J.D. (1978) Arch. Biochem. Biophys. **187**, 223-228.
11. Blobel, G., and Potter, V.R. (1967) J. Mol. Biol. **28**, 539-542.
12. Pelham, H.R.B., and Jackson, R.J. (1976) Eur. J. Biochem. **67**, 247-256.
13. Roberts, B.E., and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. USA **70**, 2330-2334.
14. Mori, M., Miura, S., Tatibana, M., and Cohen, P.P. (1981) J. Biol. Chem. **256**, 4127-4132.
15. Laemmli, U.K. (1970) Nature **227**, 680-685.
16. Bonner, W.M., and Laskey, R.A. (1974) Eur. J. Biochem. **46**, 83-88.
17. Takesue, Y., and Sato, R. (1968) J. Biochem. (Tokyo) **64**, 873-883.
18. Mori, M. (1984) Cell Tech. **13**, 238-246.
19. Tyler, D.D. (1975) Biochem. J. **147**, 493-504.
20. Lieman-Hurwitz, J., Wolf, D., Goldman, D., and Groner, Y. (1981) Biochem. Int. **3**, 107-115.
21. Sherman, L., Dafni, N., Lieman-Hurwitz, J., and Groner, Y. (1983) Proc. Natl. Acad. Sci. USA **80**, 5465-5469.