CELL-FREE SYNTHESIS OF HUMAN Cu/Zn-SUPEROXIDE DISMUTASE

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1. Introduction

Superoxide dismutase (EC 1.15.1.1) discovered by McCord and Fridovich [1] is the only protein known to catalyse the disproportionation of superoxide radicals. It may therefore be an important line of defence against toxicity arising associated with oxygen and its reduction products. Three types of superoxide dismutase are known. These have the copper/zinc, iron or manganese as the active centres. The structure, function and clinical relevance of these enzymes has been considered in [2,3]. The synthesis of the enzyme has been shown to be responsive to oxygen tension in *Streptococcus faecalis* [4], *Escherichia coli* [4–8], *Photobacterium leiognathi* [9,10], *Euglena gracilis* [11], *Saccharomyces cerevisiae* [12], rat lung [13–15] and guinea pig leucocytes [16] and to metal ion concentration in the fungus, *Dactylium dendroides* [17]. The molecular aspects of the regulation of superoxide dismutase are unknown. A basic understanding of the mechanism by which gene activity is regulated and differential gene expression is achieved can only be obtained from a detailed understanding of the messenger RNA and the synthesised polypeptide chain.

Here, we report the cell-free synthesis of the polypeptide for the human Cu/Zn superoxide dismutase in the rabbit reticulocyte lysate translation system.

2. Materials and methods

2.1. *Isolation of total RNA*

Human placenta were obtained immediately after birth, washed with ice-cold phosphate-buffered saline (pH 7.4) rapidly frozen in liquid nitrogen and stored at −70°C until required for use.

A 10–15% homogenate was prepared in 0.1 M Tris–HCl buffer (pH 9.0) containing 0.1% SDS plus 0.025 M EDTA and 0.2% diethylpyrocarbonate. Homogenization was carried out for 5 min at 4°C. An equal volume of water-saturated phenol/chloroform (1:1) was added with continuous stirring for 30 min. The mixture was then centrifuged at 3000 rev./min for 30 min. The supernatant was re-extracted with an equal volume of water-saturated phenol/chloroform mixture and centrifuged at 3000 rev./min for 30 min. Supernatants were then pooled, 2 vol. ice-cold ethanol were added and the pH of the solution adjusted to 5.0 with 4 M sodium acetate (pH 5.0). Nucleic acids were left to precipitate overnight at −20°C. The precipitate was collected by centrifugation at 20 000 × *g* for 30 min and washed with 70% ethanol.

2.2. *Oligo(dT)-cellulose chromatography*

Total RNA in 0.01 M Tris–HCl buffer (pH 7.4) containing 0.5 M NaCl, 0.5% SDS and 1 mM EDTA was loaded on a column of oligo(dT)-cellulose (Collab. Res. MA) equilibrated with the same buffer. The column was washed with the equilibrating buffer until the absorbance of the eluant at 260 nm was the same as that of the equilibrating buffer. The column was then washed with 0.01 M Tris–HCl buffer (pH 7.4) containing 0.05% SDS and 1 mM EDTA to elute bound mRNA.

2.3. *Translation*

Purified mRNA was translated in a rabbit reticulocyte lysate system containing [^{3}H]leucine. Both products were obtained from the Radiochemical Centre, Amersham. Incubations were carried out for 1 h at 30°C after which the incubation mixture was rapidly cooled. Free amino acids were separated from incorporated ones by paper chromatography using

30% methanol/10% trichloroacetic acid/10% acetic acid as the chromatographic solvent. Synthesised protein, which remains at the origin, was solubilised by heating at 50°C for 30 min with Protosol (New England Nuclear, Winchester). Incorporated radioactivity was counted on an LKB-Wallac 81000 Liquid Scintillation Counter (LKB Instr., Surrey).

2.4. *Identification of translation products*

Immediately after translation, the incubation mixture was placed on ice for 30 min and then subjected to a Laurell rocket assay [18]. The synthesised polypeptide was prepared for SDS gel electrophoresis by indirect immunoprecipitation. Rabbit antiserum specific for human Cu/Zn superoxide dismutase was prepared according to [19]. The γ-globulin function was purified by ammonium sulfate fractionation (0–50%) and chromatography on DE-32 cellulose [20]. Immunoprecipitation was carried out by addition of antibody to the translation mixture followed by incubation at 37°C for 1 h. Swine anti-rabbit immunoglobulin (DAKO immunoglobulin a/s, Denmark) was then added and a further incubation (at 4°C for 24 h) was carried out. The immunoprecipitate was pelleted by centrifugation, resuspended and washed 4 times with phosphate-buffered saline. The final precipitate was dissolved in 0.1 M Tris–acetate buffer (pH 7.4) containing 4 M urea, 1% SDS and 1% mercaptoethanol and incubated at 100°C for 10 min. The incubated sample was analysed on SDS–polyacrylamide gels according to [21]. The gels were stained, destained and sliced into 2 mm sections for radioactivity counting.

3. Results and discussion

The separation of poly(A) RNA on oligo(dT)-cellulose is shown in fig.1. The final yield was ~5 μg from 10 g frozen tissue. This agrees well with the assumption that the placenta is rich in nucleic acids. The result of a typical mRNA titration using [^{3}H]-leucine is shown in fig.2 for an incubation period of 1 h. Linearity is observed up to 2.5 μg mRNA in the translation cocktail. For this amount of mRNA, a time course for the reaction indicated linear behaviour up to 1 h. (fig.3).

The Laurell rocket assay indicates interaction of the synthesized polypeptide with the antibody to human Cu/Zn superoxide dismutase (fig.4). When the

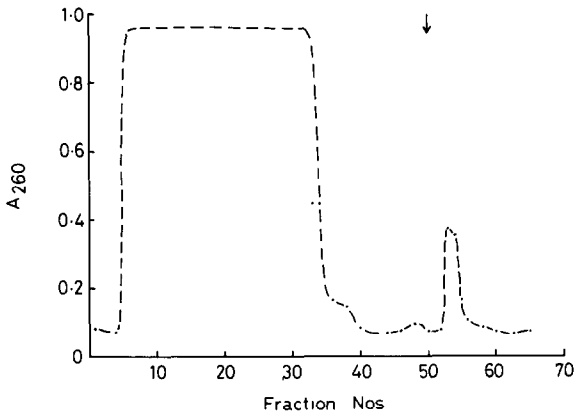

Fig.1. Purification of mRNA by oligo(dT)-cellulose chromatography. 40 A_{260} units loaded on a column equilibrated with 0.01 M Tris–HCl (pH 7.4) containing 0.5 M NaCl, 0.5% SDS and 0.1 mM EDTA. Elution with 0.01 M Tris–HCl (pH 7.4) containing 0.05% SDS and 0.1 mM EDTA is indicated by arrow.

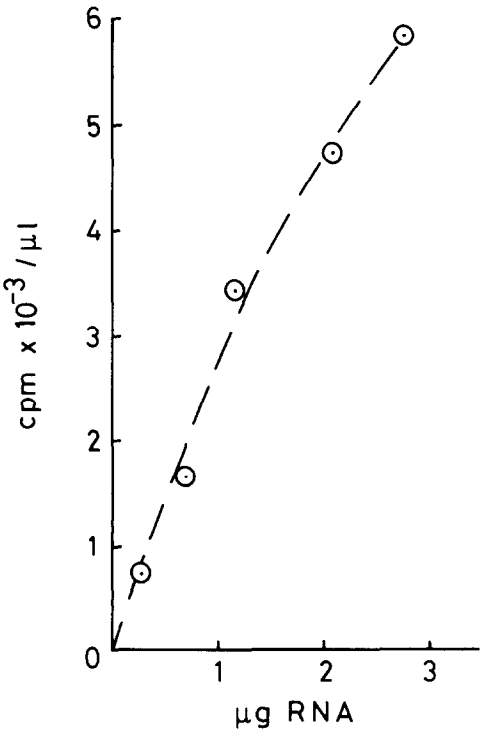

Fig.2. Effect of [mRNA] on the incoporation of [^{3}H]leucine into protein in the translation mixture. Conditions of assay as in the text.

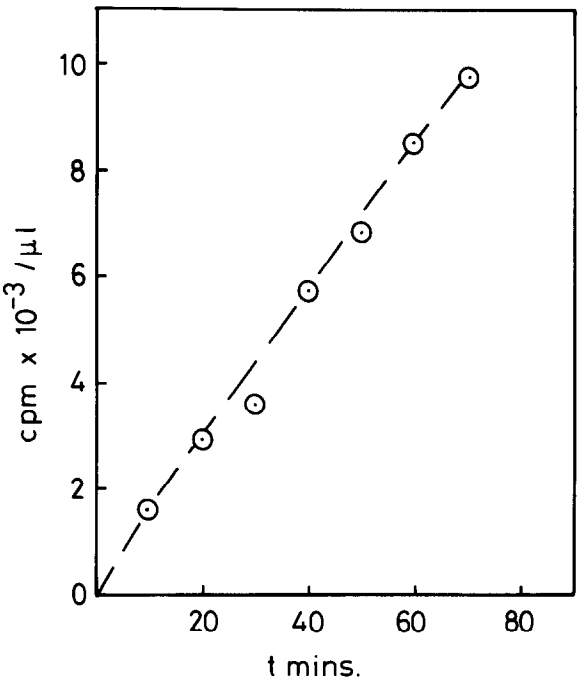

Fig.3. Time course for the incorporation of [^{3}H]leucine at 30°C at [mRNA] of 2.5 µg in the translation mixture.

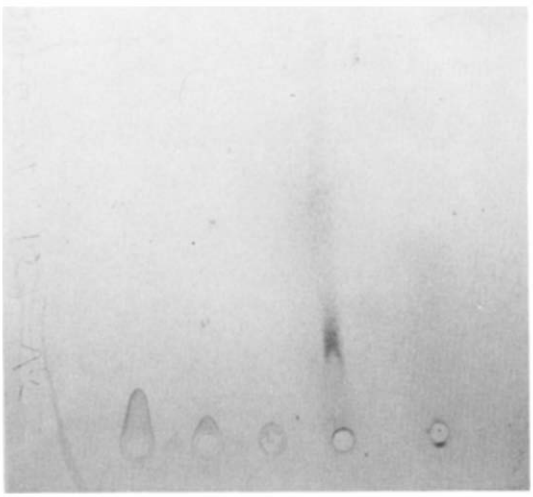

Fig.4. Laurell rocket assay of the pure protein translation mixture. Wells from left to right contain 6, 2 and 1 µg SOD/ml, translation mixture with 2.5 µg mRNA added and translation mixture without added mRNA. Translations were done for 1 h at 30°C.

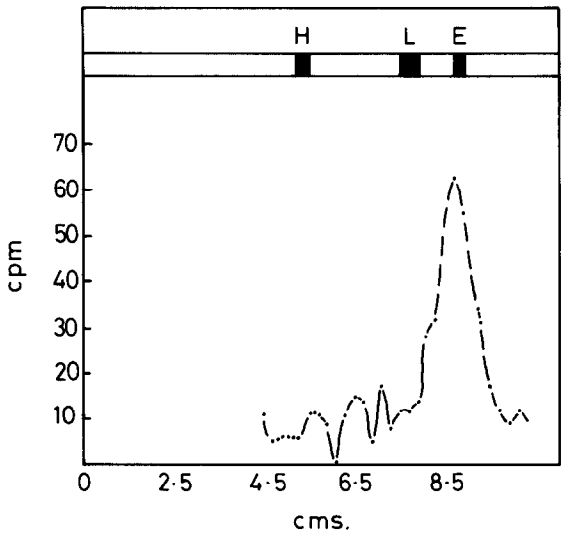

Fig.5. SDS gel electrophoresis of immunoprecipitated synthesized polypeptide chain. H and L refer to the heavy and light chains of IgG and E refers to the synthesized polypeptide chain.

immunoprecipitate was subjected to SDS gel electrophoresis after denaturation, three bands were observed (fig.5). The bands marked H and L were found to have M_r-values of 55 000 and 21 000, respectively, and they correspond to the size of the heavy and light chains of IgG whilst the faster moving band E was found to have a M_r-value of ~16 000 which corresponds to the size of the enzyme subunit. Slicing of the gel and measurement of the radioactivity indicated that radioactivity was associated mainly with this band (fig.5).

The mixture of mRNA isolated from human placenta appears to translate a polypeptide chain which has the same antigenic site and subunit size as Cu/Zn superoxide dismutase. The results obtained indicate that the human placenta is a useful tissue for the isolation of superoxide dismutase synthesising polysomes.

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