THE INDUCTION OF HISTOCHEMICALLY-DETECTABLE SUPEROXIDE DISMUTASE (Cu/Zn TYPE) BANDS ON ACRYLAMIDE GELS

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Received 27 July 1978

1. Introduction

Superoxide dismutase (SOD; EC 1.15.1.1) exists in 3 distinct forms which are characterised by the metal ions required for catalysis; Cu/Zn, Mn and Fe [1–3]. A role for these metalloproteins in protecting cells against oxygen toxicity has been postulated [4,5].

The technique of polyacrylamide gel electrophoresis has found widespread use in the study of these proteins [6–8]. Crude tissue extracts have frequently demonstrated a number of SOD activity zones on gels after electrophoretic fractionation [9–12]. However, the nature of this multiplicity has not been elucidated. We thus considered it important to determine whether these activity zones represent:

(i) Isoenzymes;
(ii) Degradation into enzymatically active components;
(iii) The association of small amounts of superoxide dismutase with other proteins.

In this communication the various properties displayed by purified superoxide dismutases from *Neurospora crassa* (both Cu/Zn and Mn-containing types) following heat treatment or pH variations are reported. We show the induction of zones of SOD activity which may be due to proteins with different net charges. In addition our results emphasise possible limitations of this technique in studying multiple forms of SOD.

2. Materials and methods

Nitroblue tetrazolium, xanthine and xanthine oxidase were purchased from BDH Chemicals Ltd, (Poole, Dorset); Bovine serum albumin was from Calbiochem, Los Angeles. CAPS [3-(cyclohexylamino)propanesulphonic acid] from Hopkins and Williams, (Chadwell Heath, Essex); ampholine carrier ampholytes pH 4–6 from LKB (South Croydon, Surrey); Potassium superoxide was from ICN Pharmaceuticals, New York. Other reagents were Analar grade.

2.1. *Biological material*

Growth of *Neurospora crassa*, wild type, and isolation of the mitochondria was essentially as in [13], with the following modifications. Fermenters, 100 l, with suitable aeration were used for growth. The hyphae were harvested 24 h after inoculation and were disrupted with a Manton-Gaulin homogeniser (APV Ltd, Crawley, Sussex).

The Cu/Zn-containing protein [22] was purified from the cytosolic fraction. It is a dimeric protein with mol. wt 31 600 (determined by gel filtration). Metal analyses showed the presence of 2 g atoms Cu/mol and 2 g atoms Zn/mol. The protein had spec. act. 3800 units/mg. One unit of activity being defined as that amount of enzyme causing a 50% decrease in the rate of nitro blue tetrazolium reduction under the conditions of the assay. The Mn-containing SOD [22] was obtained from the mitochondrial enriched fraction.

2.2. *Enzymatic analysis*

Superoxide dismutase activity was assayed by the spectrophotometric method [14] as modified [15].

The potassium superoxide method [16] was also employed.

2.3. *Electrophoretic analysis*

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing in acrylamide gels were performed at 4°C as in [17]. Gels were stained for activity by the method in [18]. SDS-gel electrophoresis was performed as in [19].

2.4. *Thermal inactivation*

The Cu-containing protein (500 units/ml) and the Mn-containing protein (200 units/ml) were heated in sealed vials, in air, in 0.1 M potassium phosphate, pH 7.8. Aliquots were withdrawn at intervals, rapidly cooled on ice and then assayed at 25°C. The effect of addition of bovine serum albumin at 1.0, 5.0 and 10.0 mg/ml on inactivation of the Cu-containing SOD was investigated.

2.5. *Effect of H^+ concentration*

The proteins were incubated at 20°C in a buffer comprising citric acid, potassium dihydrogen orthophosphate, Tris and CAPS, the pH was adjusted by the addition of HCl or NaOH. The final molarity of the buffer was 100 mM. After incubation enzymatic activity was ascertained as described above.

2.6. *Analytical ultracentrifugation*

Sedimentation velocity analyses were performed in a Beckman model E analytical ultracentrifuge equipped with Schlieren optics, in a standard 12 mm double sector and an analytical D rotor. The Cu-containing protein (max. 9.6 mg/ml) was in 50 mM Tris/100 mM KCl, pH 7.8.

3. Results

The Cu-containing protein showed a single cyanide-sensitive band of activity (on PAGE) prior to the heat treatment. Following heat treatment multiple bands of activity nearer to the anode were evident. These new bands were also inhibited by cyanide treatment (1 mM). The number of bands varied depending on the duration of heating: A maximum of 5, (3 major) being obtained after 3 h at 75°C. This multiplicity of SOD activity bands was also evident following isoelectric focusing when a maximum of 3 bands were observed. The duration of focusing (up to 24 h) did not affect the multiplicity of bands. Inclusion of bovine serum albumin during the heat treatment had no effect upon the multiplicity of bands observed. Heating caused a decrease in enzymic activity. Table 1 shows a correlation between time of heating the Cu-containing SOD and percentage activity remaining.

Storage of the Cu-containing protein at liquid N_2 temperatures at > 10 mg/ml resulted in multiple band formation. This multiple band formation was evident when the gels were stained for enzymic activity or for protein after trichloroacetate fixation.

The induction of bands of SOD activity following heat treatment or storage was not observed with the Mn-containing SOD. However, a decrease in enzymic activity upon heating was evident. Most of the activity was lost within the first 10 min heating at 75°C (see table 1).

In an attempt to explain the multiple forms of the Cu-containing SOD we carried out sedimentation analyses on the protein after storage at 77 K. Only a single symmetrical peak of material was observed when the protein was sedimented in the ultracentrifuge (rotor speed 60 000 rev./min, temp. 20°C).

Finally, incubation of the Cu-containing protein over pH 3.6–11.6 did not generate multiple SOD activity bands. Prolonged incubation at the more

Table 1
Thermal inactivation of superoxide dismutases

Protein	Duration of heating at 75°C	% Activity remaining
Cu/Zn SOD	0 time	100
Cu/Zn SOD	0.5 h	68–74
Cu/Zn SOD	1.0 h	60–63
Cu/Zn SOD	2.0 h	41–55
Cu/Zn SOD	3.0 h	16–26
Mn SOD	0 time	100
Mn SOD	10 min	< 20%
Mn SOD	45 min	0

The experiments were performed as described in the text. The results presented were obtained using the xanthine/xanthine oxidase assay. The KO_2 assay gave comparable results (see section 2.5).

acidic and basic pH ranges did however result in a decrease in enzymic activity, the activity being completely destroyed within 30 min at pH > 11.6.

4. Discussion

From this study it is evident that the purified Cu/Zn superoxide dismutase from *Neurospora crassa* can exist in several molecular forms. The multiple forms were obtained by storage of the purified protein and/or heat treatment followed by disc electrophoresis. The bands were detectable by either activity or protein staining.

The existence of multiple forms of the Cu/Zn SOD after electrophoretic fractionation has been reported [11,20]. The multiplicity has been interpreted as due either to different proteins with SOD activity or to different isoenzymes of a single protein [11]. In the present study both these explanations appear unlikely, as purified proteins were used. In attempting to explain the induction of these bands a number of possible reasons for the multiplicity are discussed.

4.1. *Proteolysis*

This appears unlikely because:

(i) The enzyme was pure, homogeneity criteria for the freshly isolated enzyme being established by normal and SDS–gel electrophoresis, isoelectric focusing in acrylamide gels and the elution of the enzyme as a single symmetrical peak during chromatography on a Sephadex G-100 column. This symmetrical peak coincided with enzyme activity and metal content.

(ii) Cu/Zn SOD is resistant to proteolysis [21].

(iii) BSA did not protect.

(iv) The sample which displayed multiple bands on acrylamide gels after storage 'modified enzyme' had the same sedimentation velocity as that of the freshly isolated protein.

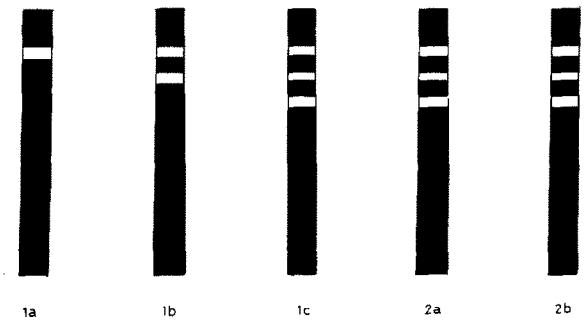

Fig.1. The effect of temperature on the dimeric Cu/Zn-containing superoxide dismutase. After heating the preparations were subjected to polyacrylamide gel electrophoresis using 10% resolving gels and 2.5% stacking gels. The running buffer was 25 mM Tris/HCl, 0.192 M glycine, pH 8.5. The gels were stained for activity by the method [18]. (1a) Original unheated sample; (1b) sample heated for 1 h; (1c) heated for 3 h. (2a) heated for 3 h with BSA at 1 mg/ml; (2b) heated for 3 h with BSA at 10 mg/ml.

4.2. *Dissociation into monomers, polymerisation or cleavage of the polypeptide chain*

This appears unlikely because:

(i) 'Modified enzyme' had the same sedimentation velocity as the sample showing a single component on acrylamide gels.

(ii) The conditions employed were not considered sufficiently rigorous to cause polypeptide chain cleavage. It is noteworthy that although the enzyme might associate with other proteins it did not bind to BSA under our experimental conditions.

4.3. *Loss of metal*

This is quite likely as it would be consistent with loss of activity and increased negative charge. This would account for all the bands obtained with the stored samples and some of the bands with the heated sample.

From this study it is not possible to establish how conformational changes could have contributed to the results obtained.

The results presented in this study have confirmed the apparently high thermal stability of the Cu/Zn containing SOD. Our results also suggested that the Mn-containing SOD is less resistant to thermal inactivation than the Cu-containing SOD isolated from the same organism.

Finally, how the electrophoretic procedures employed could have contributed to the observed multiplicity still remains to be investigated.

In summary we have demonstrated the induction of multiple forms of SOD-like activity. A difference in net charge, arising possibly from metal loss, appears

to be a major factor in the induction of these bands. It is therefore important that caution is exercised in the designation of activity zones, which are seen on acrylamide gels, as representing isoenzymes especially when studying impure preparations.

Acknowledgements

We are indebted to Dr N. G. Pon for his valuable assistance with the sedimentation velocity analyses. In addition his helpful suggestions throughout this study are gratefully acknowledged. This work was supported by the Science Research Council, England.

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