Intracellular Localization, Isolation and Characterization of Two Distinct Varieties of Superoxide Dismutase from *Neurospora crassa*

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(Received 5 September 1979)

1. *Neurospora crassa* was found to contain two distinct superoxide dismutases. 2. Most of the activity is associated with the cytosolic fraction and was shown to be the Cu/Zn-containing form of the protein. 3. Mitochondria isolated from *Neurospora crassa* showed two distinct superoxide dismutases: a cyanide-sensitive Cu/Zn-containing protein and a cyanide-insensitive form which probably contains manganese. 4. Localization experiments, using selective marker enzymes and digitonin fractionation, indicated that the cyanide-sensitive form is localized in the intermembrane space, whereas the cyanide-insensitive form is confined to the mitochondrial matrix space. 5. The cytosolic Cu/Zn-containing superoxide dismutase was isolated in high yields and extensively characterized by using e.p.r. spectroscopy, isoelectric focusing and analytical ultracentrifugation. 6. E.p.r. spectroscopy was used to monitor changes in the copper environment of the native protein after the addition of a number of potential inhibitors and after high-pH treatment. 7. Both of the cyanide-sensitive Cu/Zn-containing enzymes (cytosolic and mitochondrial) appeared to have identical properties which in turn were different from the cyanide-insensitive enzyme. 8. It is probable that the cyanide-insensitive enzyme was not previously detected, owing to its low amount (less than 10% of the total activity), greater lability than the cyanide-sensitive enzyme and the necessity of obtaining a mitochondrial-enriched fraction before its isolation.

The superoxide dismutases from a variety of organisms have been extensively studied and their physicochemical properties have been reviewed (Fridovich, 1974; Malmström *et al.*, 1975). In most eukaryotic cells there are two distinct forms of the enzyme. Firstly, and accounting for most of the activity, is a cyanide-sensitive Cu/Zn-containing protein associated with the cytosolic fraction. Secondly, a cyanide-insensitive Mn-containing protein is associated with mammalian mitochondria, presumably in the matrix space (Weisiger & Fridovich, 1973). It has also been reported that a Cu/Zn-containing superoxide dismutase is associated with mammalian mitochondria, possibly in the intermembrane space (Tyler, 1975; Peeters-Joris *et al.*, 1975). However, the localization of this latter enzyme on the outer mitochondrial membrane, possibly adsorbed to it during isolation, is still unresolved.

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Surprisingly, Misra & Fridovich (1972) and Rapp *et al.* (1973), working with *Neurospora crassa*, detected only a single superoxide dismutase, which was shown to be the Cu/Zn-containing variety. Considering the above data it seemed worthwhile to re-investigate the superoxide dismutase activity within this organism. In order to investigate the nature and the properties of the multiple forms, if present, it appeared necessary to purify and characterize the enzyme(s) from isolated mitochondria and from the cytosol. *Neurospora crassa* was ideally suited for this work, owing to the relative ease of large-scale cultivation and mitochondrial isolation.

Materials and Methods

Chemicals

DL-Isocitric acid (trisodium salt) and digitonin were obtained from Sigma Chemicals (Kingston-upon-Thames, Surrey, U.K.). NAD⁺ (free acid), ATP (disodium salt), AMP (free acid), phosphoenolpyruvate (monopotassium salt), NADH (di-

sodium salt), lactate dehydrogenase (EC 1.1.1.27, from rabbit muscle) and pyruvate kinase (EC 2.7.1.40, from rabbit muscle) were obtained from Boehringer Corp. (Lewes, Sussex, U.K.). All other reagents were of the highest quality commercially available and were obtained from BDH Chemicals (Poole, Dorset, U.K.) or Sigma Chemicals, unless otherwise specified.

Biological material and preparation of cell-free extracts

Neurospora crassa (wild-type 74A) was grown for 24 h at 30°C with 58 mM-sucrose as carbon source. Harvesting of the hyphae and preparation of the mitochondria were carried out essentially as previously described (Hall & Baltscheffsky, 1968). The hyphae were disrupted with a Manton-Gaulin homogenizer (APV, Crawley, Sussex, U.K.) at room temperature. The pre-cooled grinding medium consisted of 0.35 M-sucrose/1 mM-EDTA/0.3% (w/v) bovine serum albumin/15 mM-potassium phosphate, pH 7.8. The slurry was centrifuged at 650g for 10 min and the pellet was discarded. The supernatant was centrifuged at 15 000g for 15 min and the pellet (mitochondrial fraction) and the supernatant (cytosolic fraction) were used for purification of the superoxide dismutases.

Localization experiments

The hyphae were disrupted in the grinding medium with a rolling mincer and crushed in a mortar with a glass pestle. The slurry was filtered through muslin and centrifuged at 3000g for 10 min. The supernatant was further centrifuged at 40 000g for 5 min and the resulting pellet carefully resuspended in grinding medium and again centrifuged (as above). The washed mitochondrial pellet was finally resuspended in grinding medium to approx. 4 mg of protein/ml.

To 1 ml of the mitochondrial suspension, ice-cooled digitonin (freshly prepared in grinding medium, 0.3%, w/v) and grinding medium were added to obtain different digitonin/protein ratios in a total volume of 3 ml. The suspensions were mixed by five strokes of a Teflon pestle, transferred to a beaker and magnetically stirred for 10 min at 3°C. The mixtures were then centrifuged at 37 000g for 1 h and the supernatants were assayed for the solubilized enzymes. Control enzyme activities were determined in the intact mitochondrial pellet.

Measurements of enzyme activities

Superoxide dismutase (EC 1.15.1.1) activity was determined spectrophotometrically at 20°C. The xanthine/xanthine oxidase assay was routinely carried out as described by McCord & Fridovich (1969) as modified by Halliwell (1975). One activity unit is defined as that amount of enzyme that inhibits

by 50% the reduction of Nitro-Blue Tetrazolium under the conditions of Halliwell (1975). In addition, the potassium superoxide method was used to assay column eluates (Henry *et al.*, 1976; except that the reaction was carried out in 0.1 M-potassium phosphate, pH 7.8). The effect of cyanide on the superoxide dismutase activity was examined by the inclusion of 1 mM-KCN (pH 7.8) in the reaction mixture. This enabled us to easily distinguish between the two different types of superoxide dismutases, since the Cu/Zn-containing enzyme is inhibited by cyanide under these conditions, whereas the Mn-containing enzyme is not inhibited. NAD⁺-isocitrate dehydrogenase (EC 1.1.1.41) was assayed at 25°C by following the increase in A_{340} in a reaction mixture containing 5 mM-MnCl₂, 50 mM-DL-isocitrate, 2.5 mM-NAD⁺ and 80 mM-Tris/HCl, pH 7.5. Adenylate kinase (EC 2.7.4.3) was assayed at 25°C as described by Noda (1973). The reaction was coupled with pyruvate kinase and lactate dehydrogenase and the reaction medium consisted of 5 mM-MgCl₂, 5 mM-KCN, 5 mM-ATP, 5 mM-AMP, 5 mM-phosphoenolpyruvate, 0.2 mM-NADH, 4.0 units of pyruvate kinase, 3.6 units of lactate dehydrogenase and 60 mM-Tris/HCl, pH 7.5.

Electrophoresis

Polyacrylamide-gel electrophoresis and isoelectric focusing in polyacrylamide gels were carried out as previously described (Henry *et al.*, 1978a). The gels were stained for superoxide dismutase activity by the method of Beauchamp & Fridovich (1971) or for protein with Coomassie Brilliant Blue.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969).

Analytical ultracentrifugation

Sedimentation analyses were carried out on a Beckman model E analytical ultracentrifuge equipped with electronic speed control, an RTIC temperature control unit and schlieren optical system. An AN-D rotor was used with a 12 mm double-sector cell.

E.p.r. spectroscopy

E.p.r. spectroscopy was carried out on a Varian E-4 e.p.r. spectrophotometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.) at 77 K. All spectra are presented as the first derivative of the absorption curve. The stable radical 1,1-diphenyl-2-picrylhydrazyl was used as a g-value standard.

Other methods

Molecular weight was determined by the method of Andrews (1965) by using a Sephadex G-100 column (1.5 cm × 85 cm). Aldolase (mol.wt. 158 000), bovine serum albumin (mol.wt. 68 000),

ovalbumin (mol.wt. 45000), chymotrypsinogen A (mol.wt. 25000) and cytochrome *c* (mol.wt. 12500) were used as standards (calibration proteins, size II Combithek; Boehringer Corp.). The isoelectric points of the purified proteins were determined as previously described (Henry *et al.*, 1978a). Metal analyses were carried out on a Perkin-Elmer atomic absorption spectrophotometer, model 103. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Results

The purification procedures were conducted in 50 mM-Tris/HCl, pH 7.8 (buffer A) and at 0–4°C, unless otherwise specified.

Cytosolic superoxide dismutase

(NH₄)₂SO₄ precipitation. The homogenate was made up to 40% saturation with respect to (NH₄)₂SO₄ by addition of the solid with gentle stirring. After standing for 1 h at 4°C the precipitated material was removed by centrifugation at 23 000 *g* for 30 min and the supernatant was brought to 90% (NH₄)₂SO₄ saturation. After standing for 1 h the precipitate was collected by centrifugation and resuspended in 5 mM-Tris/HCl, pH 7.8, and dialysed against this buffer until the conductivity of the extract was 0.125 × 10⁴ μS at 4°C. During the (NH₄)₂SO₄ fractionation the pH was maintained at 7.8 by addition of NH₃ where appropriate.

Batch DEAE-cellulose. The dialysed extract from the previous step was clarified by centrifugation at 12 000 *g* for 30 min and the pellet was discarded. The supernatant was added to DEAE-cellulose (pre-swollen DE-52 micro-granular; Whatman, Maidstone, Kent, U.K.) previously equilibrated with buffer A containing 150 mM-KCl, pH 7.8, and the resulting suspension was centrifuged at 23 000 *g* for 15 min. The supernatant, which contained the cyanide-sensitive superoxide dismutase activity, was dialysed against 5 mM-Tris/HCl, pH 7.8, until the conductivity was 0.75 × 10³ μS at 4°C.

Chromatography on DEAE-cellulose. The dialysed supernatant from the previous step was applied to a column (8.0 cm × 25 cm) of DEAE-cellulose (DE-52) previously equilibrated with buffer A. After the column had been washed with the same buffer, the elution was performed with a linear gradient of 0–200 mM-KCl in buffer A. Fractions of 5 ml were collected, and their conductivity and copper contents determined. In addition, samples were assayed for superoxide dismutase activity. The active fractions were pooled and concentrated.

Chromatography on Sephadex G-100. The concentrated fraction was applied to a Sephadex G-100 column (5 cm × 53 cm). Elution was performed with

buffer A containing 100 mM-KCl at a flow rate of 15 ml/h. Fractions (5.0 ml) were collected and portions were assayed for superoxide dismutase activity and copper content. Active fractions were pooled, dialysed and concentrated.

Preparative isoelectric focusing. The preparation from the previous step was subjected to preparative isoelectric focusing in a sucrose gradient (protein concentration not exceeding 20 mg of total protein) with 1% Ampholine carrier ampholytes (w/v) (LKB, South Croydon, Surrey, U.K.) in the pH range 4–6 using a 110 ml column. The experiment was carried out as described in the LKB 8101 Instruction Manual (LKB-Producter AB, Bromma, Sweden). At the end of the experiment 1 ml fractions were collected, the pH of each fraction was determined at 4°C and portions were assayed for superoxide dismutase activity by the spectrophotometric and electrophoretic techniques.

Mitochondrial superoxide dismutases. The mitochondrial fraction was resuspended in 0.35 M-sucrose/15 mM-potassium phosphate, pH 7.8. It could be stored at 77 K for several weeks without apparent loss of superoxide dismutase activity. The mitochondria were subjected to intermittent sonication (6 × 15 s periods at 3.0–3.5 A using a Dawe Soniprobe type 1130A; Dawe Instruments, Acton, London, U.K.). An ice/NaCl bath was used to keep the temperature below 5°C during the sonication. The homogenate was clarified by centrifugation at 15 000 *g* for 15 min.

(NH₄)₂SO₄ fractionation. This was carried out essentially as described for the cytosolic superoxide dismutase (above). Proteins precipitated between 40–90% saturation were collected.

Chromatography on DEAE-cellulose. The dialysed proteins from the 40–90% (NH₄)₂SO₄ fractionation step were adsorbed on to a DEAE-cellulose (DE-23) column (5 cm × 28 cm) as previously described for the cytosolic superoxide dismutase. Elution was performed with a linear gradient of 0–300 mM-KCl in buffer A. Fractions (10 ml) were collected and the conductivity, superoxide dismutase activity and Cu and Mn contents of each fraction were determined.

The cyanide-sensitive superoxide dismutase associated with the mitochondria was eluted from the column between 40 and 60 mM-KCl in buffer A as compared with 30 and 50 mM-KCl in buffer A which had eluted the corresponding cytosolic variety. In both cases the activity peak coincided with the peak for Cu content. In contrast, a higher concentration of KCl (135–155 mM) was required to elute the cyanide-insensitive superoxide dismutase from the column. This activity peak coincided with a peak of Mn (1 μg Mn/600 units), whereas Cu, if present, was below the levels of detection.

The cyanide-sensitive superoxide dismutase was

further purified as described for the cytosolic enzyme, with chromatography on Sephadex G-100 and preparative isoelectric focusing. The cyanide-insensitive superoxide dismutase was further purified by preparative isoelectric focusing (as previously described).

Purification and criteria of purity

The purification procedures for the cytosolic cyanide-sensitive and mitochondrial cyanide-insensitive superoxide dismutases from *Neurospora crassa* are summarized in Tables 1 and 2 respectively. The specific activities of these purified proteins were 3600 and 1400 units/mg of protein respectively (using the xanthine/xanthine oxidase assay). The specific activity of the mitochondrial cyanide-sensitive Cu/Zn-containing superoxide dismutase was 3000 units/mg of protein.

The homogeneity of the three superoxide dismutase preparations was confirmed by a variety of techniques. With the freshly isolated protein a single protein band, which was enzymically active, was obtained upon electrophoresis in polyacrylamide gels under non-denaturing conditions (see Henry *et al.*, 1978b). For the electrophoretic analyses, gels stained for activity contained up to 10 units of superoxide dismutase, whereas gels stained for protein contained up to 200 µg of protein.

The enzymically active band seen with the pure protein had a similar electrophoretic mobility to the corresponding band in the crude homogenate, indicating that there was no modification during the

isolation procedure, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed a single protein species. Furthermore, homogeneity of the cytosolic cyanide-sensitive superoxide dismutase was confirmed by: (i) sedimentation velocity analyses, where a single sharp symmetrical peak was observed [the numerical value of the sedimentation constant $s_{20,w}^0$ was 2.34, which was calculated as described by Schachman (1957); this value is similar to that reported previously for the *Neurospora* enzyme (Rapp *et al.*, 1973) and for the corresponding mammalian enzyme (Hartz & Deutsch, 1969)]; (ii) its elution as a single sharp symmetrical peak from a Sephadex G-100 chromatography column, the position of elution corresponded to a protein of mol.wt. $31\,600 \pm 1500$ (three determinations) (Fig. 1). From the elution of the mitochondrial cyanide-sensitive superoxide dismutase the molecular weight was estimated to be 30800 (mean of two determinations).

Isoelectric point (pI)

The pI of the cytoplasmic Cu/Zn-containing superoxide dismutase was 4.55 ± 0.1 (six determinations), whereas the corresponding mitochondrial variety was 4.7 ± 0.08 (three determinations). These values are in close agreement with the reported values for the corresponding mammalian enzymes (e.g. Carrico & Deutsch, 1969). The pI of the mitochondrial cyanide-insensitive superoxide dismutase was appreciably higher at 5.2 ± 0.15 (three determinations).

Table 1. *Purification of the Cu/Zn-containing superoxide dismutase from the cytosol of Neurospora crassa*
Units of activity are based upon the xanthine/xanthine oxidase assay.

Step	Total volume (ml)	Total protein (mg)	Total activity (munits)	Specific activity (units/mg of protein)	Yield (%)
Crude homogenate	11 200	220 000	5500	25	100
(NH ₄) ₂ SO ₄ fractionation	2350	27 600	4600	166	84
Batch DEAE-cellulose chromatography	3750	11 000	3600	327	65
2nd DEAE-cellulose chromatography	240	1850	2200	1200	40
Sephadex G-100	60	374	748	2000	14
Isoelectric focusing	—	—	—	3600	—

Table 2. *Purification of the cyanide-insensitive superoxide dismutase from a mitochondrial enriched fraction of Neurospora crassa*
Units of activity are based upon the xanthine/xanthine oxidase assay.

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
Mitochondrial homogenate	1160	17400	232 000	13	100
40–90% (NH ₄) ₂ SO ₄ fractionation	500	2400	184 000	77	79
DEAE-cellulose (DE-23) column chromatography	350	268	105 000	391	45
Preparative isoelectric focusing	—	—	—	1400	—

Metal content

The cytosolic and the mitochondrial cyanide-sensitive superoxide dismutases were found to contain 2 mol of Cu (1.94 ± 0.14 , five determinations) and 2 mol of Zn (1.95 ± 0.1 , five determinations) per mol of enzyme (mol.wt. 31600). Assays for Fe and Mn on these proteins indicated that these elements, if present, were below detection limits. The mitochondrial cyanide-insensitive enzyme was not purified in sufficient quantity to permit atomic absorption spectroscopy.

Intracellular localization of superoxide dismutases in *Neurospora crassa*

On polyacrylamide gels the crude mitochondrial fraction routinely showed two distinct bands of superoxide dismutase activity (see Fig. 2). The slower migrating band was insensitive to 1 mM-KCN, whereas the other was inhibited by cyanide at this concentration. From the intensity of the staining zones it appeared that approx. 50% of the superoxide dismutase associated with the mitochondrial fraction is attributable to the cyanide-insensitive variety (see Arron *et al.*, 1976).

Fig. 3 shows the activities of adenylate kinase, NAD⁺-isocitrate dehydrogenase and both the cyanide-sensitive and cyanide-insensitive superoxide dismutases released as a function of digitonin concentration, relative to that of the mitochondrial protein. Both the NAD⁺-isocitrate dehydrogenase (a marker enzyme for the matrix space) and the cyanide-insensitive superoxide dismutase showed a similar pattern of release from *Neurospora* mitochondria. This indicates that this superoxide dis-

Fig. 2. Electrophoretic analysis of the types of superoxide dismutases in different fractions of *Neurospora crassa*

All gels were stained for superoxide dismutase activity in the presence or absence of 1 mM-KCN, as described in the text. Gels left to right: (i) crude mitochondrial fraction (5 units); (ii) crude mitochondrial fraction (3 units) with KCN; (iii) crude homogenate, cytosolic fraction (7 units); (iv) crude homogenate, cytosolic fraction (10 units) with KCN.

Fig. 1. Molecular-weight estimation of the Cu/Zn-containing superoxide dismutase from *Neurospora crassa* by gel filtration on Sephadex G-100

Each standard protein (3 mg) was applied to the column in a total volume of 0.5 ml, and the experiments were carried out as described in the text. V_e , elution volume; V_0 , void volume.

Fig. 3. Digitonin fractionation of *Neurospora crassa* mitochondria

Mitochondrial enzymes present in 37000g supernatant as a function of digitonin concentration. Enzyme activities are expressed as units solubilized/3.8 mg of mitochondrial protein. Units are defined as follows: adenylate kinase ($\square$), mmol of ADP produced/min; NAD⁺-isocitrate dehydrogenase ($\circ$), mmol of NAD⁺ reduced/min; superoxide dismutase, cyanide-sensitive ($\blacksquare$) and cyanide-insensitive ($\bullet$), as defined in the text.

mutase is confined to the mitochondrial matrix space.

In contrast, the cyanide-sensitive superoxide dismutase can be seen to be released at much lower digitonin/protein ratios. This pattern of release is similar to that observed with adenylate kinase (a soluble intermembrane-space enzyme) and indicates that both enzymes have the same intracellular localization.

Effect of inhibitors on e.p.r. spectrum and activity

In Fig. 4a the e.p.r. spectrum of the cytosolic superoxide dismutase is shown. The spectrum indicates Cu(II) and is very similar to that of mammalian superoxide dismutase (erythrocyte) (Carrico & Deutsch, 1969) and confirms that the

enzyme is of the Cu/Zn type. The g -values $g_{\parallel} = 2.26$ and $g_m = 2.089$ are in agreement with those reported for the *Neurospora* enzyme by Misra & Fridovich (1972) and Rapp *et al.* (1973).

The e.p.r. spectrum of the protein is perturbed by compounds that bind at or near the copper site. KCN (10mM) (Fig. 4b) caused an enhancement of the superhyperfine splitting due to nitrogen. NaN_3 (Fig. 4c) caused shifts in the g -values and a change in the Cu nuclear hyperfine coupling. Treatment with alkali (Fig. 4d) also enhanced the nitrogen hyperfine splitting. All of these effects are similar to those seen in bovine erythrocyte superoxide dismutase by Fee & Gaber (1972) and Rotilio *et al.* (1971). The effects of these treatments on the e.p.r. spectral parameters are summarized in Table 3.

The copper-chelating agent diethyldithiocarbamate (10mM) caused a small shift of g_m to higher field (Fig. 4e). At higher concentration (300mM) the g_m shifted further to 2.056 with a disappearance of $g_{\parallel}$ features (data not shown). These effects may be associated with removal of copper from the enzyme.

Table 4 shows the effect of the various compounds on the enzymic activity of the Cu/Zn-containing superoxide dismutase from the cytosol.

Fig. 4. E.p.r. spectrum of *Neurospora crassa* Cu/Zn superoxide dismutase, and the effects of various treatments

All samples except (d) were prepared at pH 7.8. (a) Untreated enzyme; (b) with 10mM-KCN; (c) with 10mM- NaN_3 ; (d) adjusted to pH 12.2 with NaOH; (e) with 10mM-diethyldithiocarbamate. Spectra were recorded at 77K with the following instrument settings: microwave power, 20mW; frequency, 9.165 GHz; modulation amplitude, 1 mT; frequency, 100kHz.

Table 3. E.p.r. characteristics of *Neurospora crassa* Cu/Zn-containing superoxide dismutase and the effect of inhibitors and high pH

The g values are the principal apparent g -values measured from the spectra (no simulations were performed). $A_{\parallel}$ is the average hyperfine splitting (in gauss) due to ^{63}Cu and ^{65}Cu along the same axis as $g_{\parallel}$. g_m is the "crossover point" of the derivative spectrum (i.e. maximum absorption).

Sample and treatment	$A_{\parallel}$ (mT)	$g_{\parallel}$	g_m
Native (pH 7.8)	13.3	2.260	2.089
Native + 10mM-KCN	19.0	2.200	2.047
Native + 10mM- NaN_3	16.3	2.234	2.041
Native (pH 12.2)	16.7	2.256	2.042

Table 4. Effect of inhibitors on *Neurospora crassa* Cu/Zn-containing superoxide dismutases

The experiments were carried out as described in the text. Superoxide dismutase activity was determined by the potassium superoxide method. The values given are the means ($\pm$ s.e.m.) of three determinations.

Inhibitor	Concentration of inhibitor (mM)	Inhibition (%)
KCN (pH 7.8)	1.0	80 (± 4)
	3.0	92 (± 6)
Diethyldithiocarbamate	1.0	56 (± 5)
	3.0	75 (± 2)
NaN_3	1.0	0
	10.0	0

The Cu/Zn enzyme associated with the mitochondrial membrane was also tested, and gave identical results. Both cyanide and diethyldithiocarbamate were potent inhibitors of enzymic activity, the latter at concentrations lower than those that affected the e.p.r. spectrum noticeably. Azide, despite its effects on the e.p.r. spectrum, was not inhibitory at the concentrations used. None of the compounds had any effect on the activity of the superoxide dismutase isolated from the mitochondrial matrix.

Discussion

From this study it is evident that *Neurospora crassa* contains two (and possibly three) distinct superoxide dismutases. The greater part of the activity (>90%) was associated with the cytosolic fraction and was shown to be the Cu/Zn-containing form of the protein. The mitochondria contain both a cyanide-sensitive Cu/Zn-containing form of the enzyme and a cyanide-insensitive variety. The electrophoretic mobility, isoelectric point, resistance to KCN, NaN₃ and diethyldithiocarbamate (at the concentrations tested) and the presence of Mn, found in parallel with enzyme activity, strongly suggest that this latter enzyme is the Mn-containing variety.

There have been a number of reports concerning the intracellular localization of the mitochondrial cyanide-sensitive superoxide dismutase using animal mitochondria (e.g. Tyler, 1975). In contrast, there is a dearth of information concerning the intracellular localization of this enzyme in fungal and higher plant mitochondria (see Arron *et al.*, 1976; Jackson *et al.*, 1978). This is attributable to the absence of good enzyme markers for the mitochondrial intermembrane space using organelles from these sources.

In contrast with the frequently used technique of differential swelling to obtain information on the intracellular localization of superoxide dismutases we have employed a different approach, based upon digitonin fractionation of isolated *Neurospora* mitochondria. The results obtained are consistent with reports, in which mammalian mitochondria were used, that a cyanide-sensitive superoxide dismutase is localized within the mitochondrial intermembrane space. The activity of the cyanide-insensitive superoxide dismutase was latent, being released only upon disruption of the matrix space. It may be possible to use the KCN-insensitive superoxide dismutase as an additional marker enzyme for the mitochondrial matrix space after further characterization.

In our studies both the cytosolic and mitochondrial Cu/Zn-containing proteins appeared similar, showing only small differences in electrophoretic mobilities in polyacrylamide gels under non-denaturing conditions. This may reflect differences in the charge of the protein molecule, possibly arising from variations in their metal content.

The preparative procedures described for the cytosolic Cu/Zn-containing superoxide dismutases consistently yielded highly purified proteins in quantities sufficient for physicochemical characterization. Here we have used the technique of e.p.r. spectroscopy to monitor changes in the environment of the Cu after various treatments. Although this approach has previously been applied to the mammalian Cu/Zn-containing superoxide dismutase, this study represents the first attempt to gain such information about the Cu environment of this enzyme isolated from non-mammalian sources.

It has previously been reported that the Cu atoms (2/mol) are directly involved in catalysis, undergoing alternate oxidation and reduction (see Fridovich, 1978). The changes in the e.p.r. spectral characteristics after the various treatments were correlated with their effect upon enzyme activity. In addition to being potent inhibitors of enzyme activity, cyanide and diethyldithiocarbamate were seen to alter the e.p.r. spectra of the cytosolic Cu/Zn-containing superoxide dismutase; this suggests a direct effect on the Cu environment. The inhibitory effect of diethyldithiocarbamate on mammalian (whole rats) Cu/Zn-containing superoxide dismutase was previously reported (Heikkilä *et al.*, 1976); we have confirmed this effect using superoxide dismutase isolated from a non-mammalian source, but have also demonstrated a direct effect of this reagent on the Cu environment of the protein. Similarly, high-pH treatment was seen to alter the Cu environment of the protein, correlating with a rapid loss of enzyme activity (Henry *et al.*, 1978b).

Although azide at the concentrations tested had no effect on the enzyme activity of the Cu/Zn-containing superoxide dismutase, it did alter the e.p.r. spectral parameters. Fee & Gaber (1972) presented evidence that azide binds to the Zn (2/mol in the native Cu/Zn-containing enzyme), which is not directly involved in catalysis.

The purification procedure for the cyanide-insensitive enzyme obtained from isolated mitochondria gave homogeneous preparations, as detected by polyacrylamide-gel electrophoresis. Unfortunately, this enzyme was not present in sufficient quantities for further detailed characterization.

In the studies of Misra & Fridovich (1972) the *Neurospora crassa* Cu/Zn-containing superoxide dismutase was purified by a rather drastic method (Tsuchihashi, 1923) involving extraction by organic solvents. Rapp *et al.* (1973) used less-drastic chromatographic techniques in aqueous solutions, but used a preliminary heat-treatment at 75°C for 10 min. It is probable that organic solvents and heat-treatment could have affected the stability of the cyanide-insensitive enzyme. Indeed, we have previously reported that heat-treatment of the cyanide-insensitive Mn-containing superoxide dis-

mutase causes rapid inactivation, less than 20% of the original activity remaining after 10 min at 75°C (Henry *et al.*, 1978b).

We thank Dr. K. K. Rao, Dr. B. Halliwell, Dr. N. G. Pon and Mrs. J. Farmer for their helpful discussions and assistance. This work was supported by the Science Research Council (U.K.).

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