Absence of the iron-containing superoxide dismutase in mitochondria from mustard (*Brassica campestris*)

Marvin L. SALIN and Susan M. BRIDGES

Department of Biochemistry, Mississippi State University, Mississippi State, MS 39762, U.S.A.

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Mitochondria were isolated from mature leaves as well as etiolated seedlings of *Brassica campestris* (mustard), a eukaryote previously shown to possess the iron-containing isoenzyme of superoxide dismutase. On the basis of KCN- and H₂O₂-sensitivity, and on polyacrylamide-gel analysis, only the cuprozinc and manganous superoxide dismutases were found in mitochondria. The iron-containing enzyme was absent. The manganous enzyme was found in the mitochondrial matrix, whereas the cuprozinc enzyme appeared to be localized in the intermembrane space.

Superoxide dismutases are a class of metallo-enzymes that catalyse the dismutation of two superoxide anions as shown in the following equation:

The enzymes, ubiquitously found in all aerobic organisms, have been purified from several sources and classified into three groups according to metal content: cuprozinc, manganous and iron-containing superoxide dismutases (Fridovich, 1975). The cuprozinc enzyme is inhibited by cyanide, whereas the manganous and iron enzymes are cyanide-resistant. The cuprozinc enzyme is found mainly in eukaryotes, whereas the manganous enzyme has been isolated from both prokaryotic and eukaryotic sources (Fridovich, 1975). The iron enzyme was previously thought to be restricted to prokaryotes (Asada & Kanematsu, 1978). More recently, however, reports have appeared of the iron enzyme in the eukaryotic protozoon *Euglena* (Kanematsu & Asada, 1979; Lengfelder & Elstner, 1979). Moreover, we have isolated and characterized an iron-containing superoxide dismutase from leaves of mustard (*Brassica campestris*) (Salin & Bridges, 1980a). The enzyme was found in the chloroplast stroma of mustard leaves (Salin & Bridges, 1980b). In the present paper we report the results of our examination of mitochondria isolated from mustard leaves as well as etiolated (achloroplastic) mustard seedlings in order to further determine localization of superoxide dismutase isoenzymes in organelles of plants known to contain the iron enzyme.

Materials and methods

Cytochrome *c* (Type III), xanthine oxidase (Grade I) and Nitroblue Tetrazolium (Type III) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Percoll was purchased from Pharmacia, Piscataway, NJ, U.S.A. Other chemicals were Reagent grade. Mustard (*Brassica campestris*) leaves were field-grown locally. Etiolated seedlings were grown on vermiculite in a dark chamber and harvested 5 days after sowing.

Mitochondria from leaves and etiolated seedlings were isolated and purified on a Percoll gradient as described by Jackson *et al.* (1979). Outer membranes of mitochondria were disrupted by hypotonic treatment as follows. Mitochondria were pelleted and resuspended in 10 mM-Mops (4-morpholinepropanesulphonic acid), pH 7.4, stirred at 0°C for 10 min and then centrifuged at 12000 *g* for 10 min. The pellet was resuspended in wash media containing 30 mM-Mops, pH 7.4, 1 mM-EDTA and 300 mM-mannitol. Alternatively, membranes were disrupted by the addition of digitonin to intact mitochondria (Day & Wiskich, 1975). Mitochondrial protein content was determined and appropriate amounts of digitonin suspended in 50% (v/v) ethanol were added. Control mitochondria received a portion of the ethanol solution minus the digitonin. After 30 min stirring at 0°C, the suspensions were spun at 11000 *g* for 15 min. The pellet, containing mitochondria, was resuspended in wash media and assayed for various enzyme activities. The supernatant was retained and also assayed for various enzymes.

Superoxide dismutase (EC 1.15.1.1) activity was assayed by the cytochrome *c*-reduction method (McCord & Fridovich, 1969). One unit of activity is defined as the amount of enzyme required to inhibit cytochrome *c* reduction by 50%. Superoxide dismutase was routinely assayed in the presence of 10 μ M-KCN to inhibit cytochrome oxidase. This low cyanide concentration is insufficient to inhibit the cuprozinc enzyme. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed as described by Wharton & Tzagoloff (1967), malate dehydrogenase (EC 1.1.1.37) by the method of Ochoa (1955), and antimycin A-insensitive NADH cytochrome *c* reductase as described by Day *et al.* (1979). Enzymic assays were performed in the mitochondrial resuspension medium and then in resuspension medium containing 0.05% Triton X-100 added to rupture membranes.

Analytical electrophoresis was performed on 7.5% (w/v) polyacrylamide gels as described by Davis (1964). Superoxide dismutase activity on polyacrylamide gels was localized by using the photochemical method of Beauchamp & Fridovich (1971). Gels were scanned with a Perkin-Elmer model 552-0800 integrating gel scanner.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard, and chlorophyll was determined in 80% (v/v) acetone by the method of Vernon (1960).

Results

Crude homogenates of *Brassica campestris* leaves as well as etiolated (achloroplastic) seedlings electrophoresed on 7.5%-polyacrylamide gels and stained for superoxide dismutase activity exhibited at least three distinctive types of bands (Fig. 1). The band closest to the cathode (1) was insensitive to cyanide and 2 mM- H_2O_2 , indicating that it was either a

mangano superoxide dismutase (Asada *et al.*, 1975) or a mangano protein with superoxide dismutase-like activity (Lumsden & Hall, 1975). The second band (2) was resistant to cyanide, but sensitive to H_2O_2 , and had an R_f identical with that of the iron isoenzyme of superoxide dismutase we have recently characterized (Salin & Bridges, 1980a). The most anodic group of bands exhibited the cyanide-sensitivity of the cuprozinc class of superoxide dismutases. All bands of enzymic activity were destroyed by boiling the extract for 5 min.

Fig. 1. Scan of superoxide dismutase-activity-stained gel electrophoretograms obtained with an extract of mustard seedlings

Etiolated seedlings were ground in 50 mM-potassium phosphate buffer, pH 7.8, containing 0.2% (v/v) Triton X-100. After centrifugation at 10000 *g* for 10 min, the homogenate was placed on top of 7.5% (w/v) polyacrylamide gels. Electrophoresis and staining were as described in the Materials and methods section. (a) Control; (b) 2 mM-KCN contained in staining mixture; (c) 2 mM- H_2O_2 contained in staining mixture. Bands 1-3 are referred to in the text.

Table 1. Characteristics of intact mitochondria isolated from mature mustard leaves

Enzymic analysis, protein and chlorophyll determinations were performed as described in Materials and methods section. Values were compared with those obtained after membrane rupture by the addition of 0.2% (v/v) Triton X-100. Values are from one representative experiment, with little variation observed in five separate separations.

Activity in intact mitochondria (% of total activity)	
Cytochrome oxidase	17
Malate dehydrogenase	22
Chlorophyll (μ g/mg of protein)	
	19
Superoxide dismutase activity (unit/mg of protein)	
Cyanide-sensitive	0.5
Cyanide-resistant	0.8

We have previously shown that the iron-containing superoxide dismutase is found in the chloroplast stroma (Salin & Bridges, 1980b) and is readily released on membrane rupture. In an effort to learn more about isoenzymes of superoxide dismutase found in mitochondria from plants that contain the iron enzyme, we prepared mitochondria from both mature green mustard leaves and etiolated mustard seedlings. Table 1 summarizes some of the characteristics of the mitochondrial fraction obtained from mature green leaves. As revealed by the latency of marker enzymes, cytochrome oxidase and malate dehydrogenase, the mitochondria appeared to be about 80% intact. Contamination by chlorophyll was apparent, albeit small in extent. Cyanide-resistant superoxide dismutase activity appeared to be slightly more abundant in mitochondria than the

cyanide-sensitive cuprozinc enzyme. Fig. 2 shows activity-stained-polyacrylamide-gel scans for mitochondria from mature green leaves. In contrast with the observation with crude extracts, only two types of bands were observed for mitochondria. One type was sensitive to KCN (cuprozinc enzyme) and the other was H_2O_2 - and KCN-resistant (mangano enzyme or protein). The iron enzyme, if present, would occupy a position on gels between the cuprozinc enzyme and the mangano enzyme (Fig. 1, band 2). The lack of KCN-resistant, yet H_2O_2 -sensitive, activity at the R_F corresponding to the iron enzyme, would argue against the presence of an iron-containing superoxide dismutase in mitochondria from mustard leaves.

Similar observations were recorded for mitochondria isolated from etiolated mustard seedlings, where chloroplast contamination would not be a factor. Fig. 3 shows that KCN once more

Fig. 2. Scan of polyacrylamide-gel electrophoretograms obtained with a mitochondrial extract of mustard leaves. Conditions were as described in the Materials and methods section. (a) Standard staining mixture; (b) stained in the presence of 2 mM-KCN; (c) stained in the presence of 2 mM- H_2O_2 .

Fig. 3. Scan of polyacrylamide-gel electrophoretograms obtained with a mitochondrial extract of etiolated mustard seedlings.

Conditions were identical with those for Fig. 2.

diminished activity in two of the bands. The remaining band was both KCN- and H_2O_2 -resistant. Again, no evidence could be found on the basis of either H_2O_2 -sensitivity or R_F values for the iron-containing superoxide dismutase. Mitochondria obtained from etiolated mustard seedlings were found to contain only trace amounts of 6-phosphogluconate dehydrogenase, a presumed etioplast marker (Schnarrenberger *et al.*, 1975). We conclude, therefore, that our mitochondria were relatively free of etioplast contamination.

In an effort to determine approximate localization of various superoxide dismutase isoenzymes within mitochondria from etiolated mustard seedlings, intact organelles were incubated with various concentrations of digitonin. Rupture of the mitochondrial outer membrane was monitored during the course of titration by assaying antimycin A-insensitive NADH:cytochrome *c* oxidoreductase (Day & Wiskich, 1975). In addition, intactness of the outer membrane was determined by assaying cytochrome *c* oxidase. The mitochondrial outer membrane is impermeable to cytochrome *c*, and therefore oxidation of this protein would be a reflection of rupture of the membrane barrier (Quail, 1979). Inner-membrane intactness was monitored by assaying the matrix marker, malate dehydrogenase. Fig. 4 shows the results from a representative experiment. As can be seen, release of the cuprozinc

enzyme closely paralleled the rupture of mitochondrial outer membranes, thereby indicating this isoenzyme is predominantly localized in the intermembrane space. In contrast, the release of mangano protein closely paralleled matrix-marker activity. Addition of large amounts of detergent solubilized matrix markers as well as the mangano enzyme. We can conclude, therefore, that the mangano enzyme is confined to the matrix, whereas the cuprozinc enzyme is localized in the intermembrane space.

Discussion

The present results show that the iron-containing superoxide dismutase was found in crude extracts from mustard leaves, but was not present in the mitochondria. Although we previously reported finding the iron enzyme associated with the mitochondrial fraction from mustard leaves (Salin & Bridges, 1980a), we now believe this was the result of chloroplast contamination, since the enzyme was absent in highly purified mitochondria obtained from mustard leaves or etiolated seedlings. We have clearly demonstrated the iron-containing superoxide dismutase in the chloroplast stroma of *Brassica campestris* (Salin & Bridges, 1980b). The enzyme was readily released on rupture of the chloroplast envelope. It would appear, therefore, that in mustard, the iron-containing superoxide dismutase is restricted to the chloroplast stroma, whereas only the cuprozinc and mangano enzyme are found in the mitochondria. Our finding of the mangano enzyme confined totally to the matrix, as well as the cuprozinc enzyme present in the intermembrane space, is in agreement with previous reports (Weisiger & Fridovich, 1973; Jackson *et al.*, 1978; Henry *et al.*, 1980).

The lack of iron-containing superoxide dismutase in mitochondria is most noteworthy with respect to the evolution of organelles. The question arises as to why chloroplasts from *Brassica campestris* contain an iron enzyme, whereas neither mitochondria from the same organism nor chloroplasts from other genera contain the isoenzyme. In order to gain further insight into evolutionary trends of the iron superoxide dismutase in photosynthetic organisms, a survey of various families of vascular plants for superoxide dismutase isoenzymes is required.

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Fig. 4. Release of membrane-bound and matrix markers from mitochondria obtained from etiolated mustard seedlings

Mitochondria were prepared, treated, and enzymes assayed as described in the Materials and methods section. Values represent the percentages of total activities recorded. Assays were performed in mitochondrial resuspension medium, followed by mitochondrial resuspension medium containing 0.05% Triton X-100. ●, Antimycin A-insensitive NADH:cytochrome *c* reductase; ○, cytochrome *c* oxidase; ★, malic dehydrogenase; ■, cuprozinc superoxide dismutase; □, mangano superoxide dismutase.

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