INTRACELLULAR DISTRIBUTION OF MANGANESE AND FERRIC SUPEROXIDE DISMUTASES IN BLUE–GREEN ALGAE

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1. Introduction

On the basis of the metal in the enzyme, three types of superoxide dismutases have been isolated from organisms that range from anaerobic prokaryotes to higher organisms which represent different stages of evolution. Cu,Zn-superoxide dismutase has been found in vertebrates, land plants and fungi. However, prokaryotes, protozoa and most algae lack the Cu,Zn-enzyme, but contain Fe- and/or Mn-superoxide dismutases [1,2]. The Mn-enzyme also has been found in mitochondrial matrices from yeast, plants and mammals [3–5]. Although anaerobic bacteria contain only the Fe-enzyme [6–8], *Escherichia coli* [9] and *Euglena gracilis* [10] have both the Mn- and Fe-enzymes.

We have shown that the blue–green alga, *Plectonema boryanum*, also contains both Fe- and Mn-superoxide dismutases [11]. Because manganese is essential for the photosynthetic evolution of oxygen, it is important to determine the intracellular localization of Mn-superoxide dismutase in blue–green algae (Cyanobacteria), the most primitive algae with photosystem II. We report here the localization of Mn-superoxide dismutase in the thylakoids and that of the Fe-enzyme in the cytosol of three species of blue–green algae: *P. boryanum, Anabaena variablilis* and *Anacystis nidulans*. A part of this study has appeared in preliminary form [12].

2. Materials and methods

Cells of the three species of blue–green algae were cultured as in [11], with the following modifications: KNO_3 concentration was decreased in a culture medium to 1 g/l and 1% CO_2 was bubbled through the culture at 25°C. The algal cells were harvested after 6 days of culture and washed with 1 mM EDTA. The cells cultured in a medium containing 3 g KNO_3/l were resistant to lysozyme, and prolonged incubation was required for the preparation of spheroplasts. This is why we used a culture medium containing a low concentration of KNO_3.

Spheroplasts were prepared and cell fractionation was done as follows [13]; washed cells (0.5 g fresh wt) were incubated at 30°C for 3 h with 0.1% lysozyme dissolved in 50 ml incubation medium that contained 0.6 M sucrose, 30 mM sodium phosphate (pH 6.8) and 10 mM $MgCl_2$. Spheroplasts were collected by centrifugation at 5000 × *g* for 10 min, then were washed once with the incubation medium. Washed spheroplasts were sonicated for 2–3 s at 20 kHz after suspending them in 1 or 2 ml of the incubation medium. Unbroken cells were removed by centrifugation at 8000 × *g* for 10 min and the supernatant was further centrifuged at 30 000 × *g* for 60 min. The sediment suspended in the incubation medium is referred to as the thylakoids and supernatant is as the cytosol. To avoid cross-contamination, we centrifuged again each fraction at 30 000 × *g* for 30 min; the pellet or supernatant was used. The isolated thylakoids had Hill reaction activity of 100–150 μmol O_2 evolved mg $chl^{-1}.hr^{-1}$ when ferricyanide was used as the electron acceptor.

On the basis of the inhibition of cytochrome *c* reduction by O_2^- superoxide dismutase activity was assayed by a modification [14] of the procedure in [15]. Reduction of cytochrome *c* was followed from the increase in A_{550}; the reference was fixed at 540 nm

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on a Hitachi 356 dual-wavelength spectrophotometer. Results are presented in McCord-Fridovich units [15]. Protein was determined by the Lowry method [16]. The Mn contents were determined in a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer with a GA-2 graphite atomizer.

Antibody to *Plectonema* Fe-superoxide dismutase was prepared as in [1]. Xanthine oxidase and lysozyme (type I) were obtained from Boehringer and Sigma, respectively. The Fe- and Mn-superoxide dismutases were isolated from *Plectonema boryanum* as in [11].

3. Results and discussion

The antibody to *Plectonema* Fe-superoxide dismutase reacted with *Plectonema* Fe-enzyme and inhibited its enzymatic activity. In contrast, the Mn-enzyme from the same alga neither reacted with nor was inhibited by the antibody (fig.1). Thus, the Mn- and Fe-superoxide dismutases from *P. boryanum* can be distinguished immunologically. Using these properties of the antibody we tested the intracellular distribution of the Fe- and Mn-isozymes of superoxide

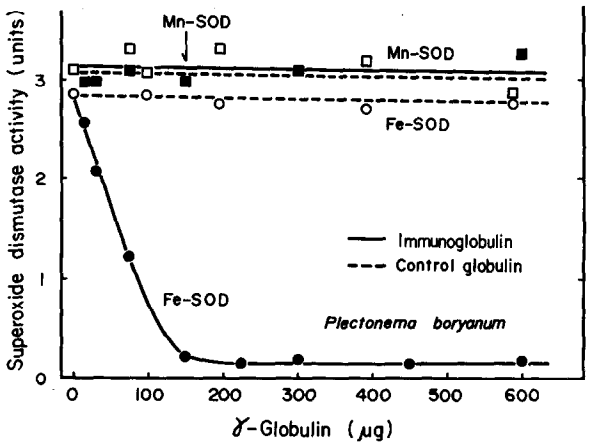

Fig.1. Effect of the antibody to the *Plectonema* Fe-superoxide dismutase on Fe- and Mn-superoxide dismutases from *Plectonema boryanum*. Superoxide dismutase (3 units) was incubated with the indicated amounts of the immuno- or control globulin in 0.15 M NaCl containing 20 mM potassium phosphate (pH 7.8) in 0.14 ml total vol. at 25°C. After 1 h incubation the enzymatic activity was determined under the standard conditions.

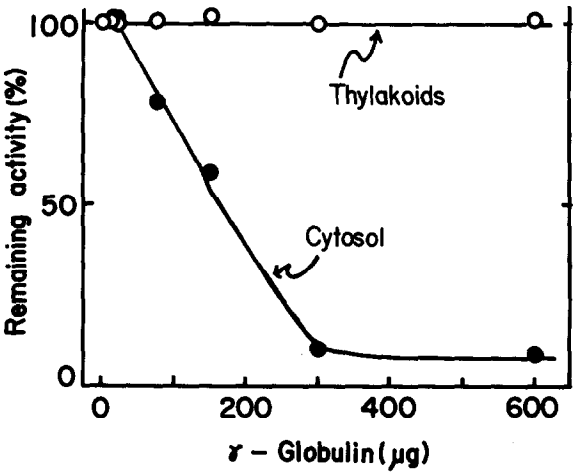

Fig.2. Effect of the anti-Fe-superoxide dismutase on *Plectonema* cytosol and thylakoid superoxide dismutases. The cytosol and thylakoid fractions containing 1–3 units of superoxide dismutase were incubated with the indicated amounts of the immunoglobulin in 0.15 M NaCl containing 20 mM potassium phosphate (pH 7.8) at 25°C for 1 h. Enzymatic activity was determined under the standard conditions. Control globulin did not affect the enzymatic activity in both the fractions.

dismutase. The antibody inhibited the enzyme in the cytosol but not in the thylakoids (fig.2); this indicates the localization of Fe-superoxide dismutase in the cytosol, and that of the Mn-enzyme in the thylakoids

The same subcellular distribution of Mn- and Fe-superoxide dismutases was observed when the cytosol and thylakoids were separated by sucrose density centrifugation after the spheroplasts had been disrupted in a hypotonic medium. Superoxide dismutase in the green thylakoid fraction was affected very little by the anti-Fe-superoxide dismutase but the enzymatic activity in the blue cytosol fraction was almost completely inhibited (fig.3).

We have shown that the Fe-superoxide dismutase of *P. boryanum* is inactivated by H_2O_2 but the Mn-enzyme is not [11]. To confirm the binding of Mn-superoxide dismutase to the thylakoids we tested the effect of H_2O_2 on the enzymatic activity in the thylakoids and the cytosol of *P. boryanum*. Cyanide was added to suppress catalase. Both the Mn- and Fe-superoxide dismutases are insensitive to cyanide [11]. As expected from the effect of the anti-Fe-superoxide dismutase, only the thylakoid enzyme was insen-

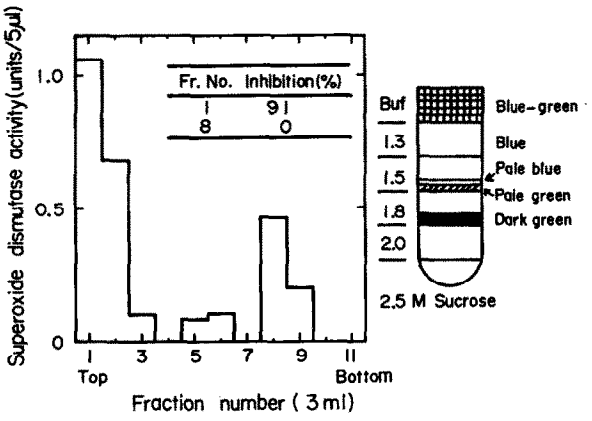

Fig.3. Effect of the anti-Fe-superoxide dismutase on *Plectonema* superoxide dismutase in cytosol and thylakoids separated by sucrose density centrifugation. *Plectonema* spheroplasts in isotonic solution (0.6 M sucrose) were disrupted by 5-fold dilution with water and were subjected to discontinuous sucrose density centrifugation as indicated at 23 000 rev./min for 3 h. The inhibition by the immunoglobulin of superoxide dismutase in the cytosol (fraction no. 1) and in thylakoids (fraction no. 8) were determined as in fig.2.

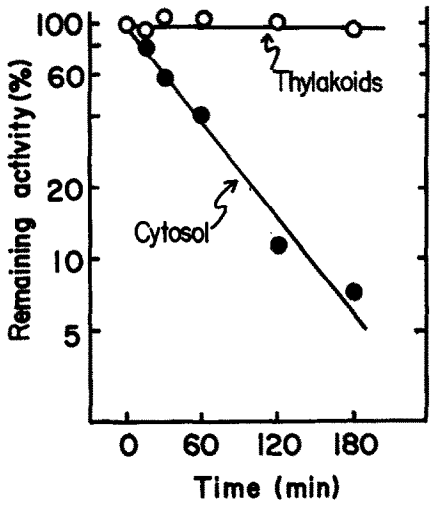

Fig.4. Effect of hydrogen peroxide on superoxide dismutase in cytosol and thylakoids. *Plectonema* cytosol and thylakoid fractions containing 1–3 units of superoxide dismutase were incubated in 0.9 mM H_2O_2 containing 1 mM KCN and 50 mM phosphate (pH 7.8) in 1 ml total vol. The enzymatic activity was determined using a small aliquot of the treated enzyme under the standard conditions at suitable intervals.

sitive to H_2O_2 (fig.4). In the absence of cyanide, no inactivation of the cytosol enzyme was observed, probably due to the decomposition of H_2O_2 with catalase. Recently the insensitivity of Mn-superoxide dismutase to H_2O_2 has been confirmed for the enzymes from *Escherichia coli* [17,18], *Rhodopseudomonas spheroides* [19], *Bacillus stearothermophilus* [20], and from a red alga [21], although the inactivation of *E. coli* Mn-enzyme by H_2O_2 was reported [22]. Inactivation of Fe-superoxide dismutases from several sources by H_2O_2 also has been corroborated [7,10,11,19,23].

The thylakoids and cytosol were separated from cells of *Anabaena variabilis* and *Anacystis nidulans*, and the effect of H_2O_2 on superoxide dismutase in both fractions was tested under the conditions as above. The cytosol enzyme from both algae was completely inactivated by H_2O_2. The *A. variabilis* thylakoid enzyme was not affected by H_2O_2 as was the case for the thylakoid enzyme from *P. boryanum*, but, the thylakoid enzyme from *A. nidulans* was partially inactivated.

The thylakoid and cytosol from the three algae were subjected to isoelectric focusing (Ampholine pH 3.5–10.0) on polyacrylamide gel discs and the superoxide dismutase activity was located according to [24] (fig.5). The thylakoids dissolved in 3% Triton X-100 were used. The cytosol enzyme from the three algae was found at a position corresponding to a pI of ~4; the activity band disappeared when the sample was incubated in H_2O_2 and cyanide before isoelectric focusing. The thylakoid enzyme gave a single band corresponding to pI values higher than those of the cytosol enzyme. Unlike the cytosol enzyme the thylakoid enzyme band did not disappear by the H_2O_2 treatment which confirms the results of fig.4. Thus, superoxide dismutase in the cytosol and the thylakoids has different pI values. The effects of anti-Fe-superoxide dismutase and of H_2O_2 indicate the localization of Fe-superoxide dismutase in the cytosol and of the Mn-enzyme in the thylakoids of blue–green algae.

The thylakoid enzyme of *P. boryanum* was not released by incubation in 5 mM phosphate or Tris–HCl (pH 6.8); in 10 mM $MgCl_2$; in 1 mM EDTA or in 0.1 M KCl containing 5 mM Tris–HCl (pH 6.8) for 1 h at 0°C with subsequent centrifugation at 56 000 × *g* for 40 min. Little activity was found in

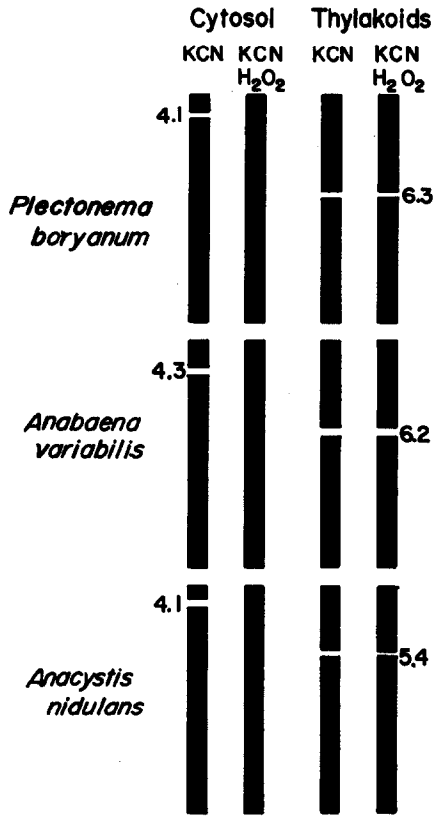

Fig.5. Isoelectric focusing pattern of superoxide dismutase in the cytosol and thylakoids of blue–green algae. The cytosol and thylakoid fractions, each containing 3–4 units of superoxide dismutase, were focused with ampholine (pH 3.5–10.0) on polyacrylamide gel disc. Thylakoids were dissolved in 3% Triton X-100 before the application. Where indicated, the sample had been incubated in 5 mM H_2O_2 containing 5 mM KCN for 12 h at 0°C. Achromatic zone indicates the enzyme and the numbers show its pI value. The pH gradient was determined by cutting the gels into 4 mm slices, eluting the slices overnight in distilled water and measuring the pH.

the supernatant; almost all being recovered in the pellets. Unlike the hypotonic medium and salt, detergents solubilized the thylakoid enzyme. When the thylakoids were incubated in 0.5% Triton X-100 and lauryldimethylamine oxide containing 30 mM phosphate (pH 6.8) for 1 h, the enzyme activity increased 2.1- and 1.6-fold, respectively. Adsorption of the Triton X-100-dissolved thylakoids on the DEAE-cellulose column, and elution with NaCl resulted in the separation of superoxide dismutase from chlorophyll (chl)-containing thylakoid membranes. Thus, the release of the enzyme from the thylakoids by the detergent causes an increase in activity which is probably due to the diffusion-controlled reaction rate of O_2^- with superoxide dismutase ($\sim 10^9$ $M^{-1}.s^{-1}$). The cytosol enzyme was not affected by Triton X-100.

The ratio of superoxide dismutase activity in the cytosol to that in thylakoids was 88:12 (*P. boryanum*) and 86:14 (*A. variabilis*) when the thylakoid enzyme was assayed in 0.5% Triton X-100. The enzyme contents in the thylakoids were 31 (*P. boryanum*) and 36 (*A. variabilis*) units.mg chl.$^{-1}$ which correspond to ~0.1 mol enzyme.400 mol chl.$^{-1}$ (photosynthetic unit^{-1}) based on spec. act. 3000 units.mg protein^{-1} and mol. wt 42 000 for Mn-superoxide dismutase. The contents of thylakoid-bound Mn were found to be 6–8 atoms.400 mol chl.$^{-1}$ by atomic absorption spectrophotometry. Mn-superoxide dismutase has been shown to contain 1 or 2 atoms of Mn per molecule; thus, Mn-superoxide dismutase in the thylakoids would account for 1.4% or 2.8% of the bound Mn.

We have shown the presence of Fe- and Mn-superoxide dismutases in *P. boryanum* [11]. The results here indicate that two other species of blue–green algae contain the Fe- and Mn-enzymes and that Fe-superoxide dismutase localizes in the cytosol and that Mn-superoxide dismutase localizes exclusively in the thylakoids of all three species. We showed the binding of Mn-superoxide dismutase to the thylakoids of Euglena [10] and of spinach chloroplasts [12]. The stroma of Euglena chloroplasts contains Fe-superoxide dismutase [10] but the spinach stroma contains Cu,Zn-superoxide dismutase [25]. Thus, the cytosol of blue–green algae and the stroma of green algae both have the Fe-enzyme and the thylakoids of both algae bind the Mn-enzyme. This is evidence of the endosymbiotic origin of the chloroplasts of the eukaryotic green alga from prokaryotic cyanobacterium which existed as an intracellular symbionant in primitive eukaryotes. It is an interesting, but unsolved question why the Fe-superoxide dismutase in the stroma of algal chloroplasts is replaced by Cu,Zn-superoxide dismutase in the stroma of higher plants.

The superoxide dismutase in blue–green algae protect the cells from the deleterious effects of O_2^- which is produced in thylakoids on illumination through autoxidation of the primary electron accep-

tor in photosystem I [26,27]. Since Mn is essential for the evolution of oxygen, the binding of Mn-superoxide dismutase to the thylakoids suggests that the dismutase participates in the water–oxidation system. However, it is premature to conclude so because apparent contents of Mn-superoxide dismutase are low in terms of the photosynthetic unit and of the thylakoid-bound Mn.

Acknowledgements

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