Superoxide Dismutase and Catalase in the Protection of the Proton-Donating Systems of Nitrogen Fixation in the Blue-Green Alga *Anabaena cylindrica*

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1. Superoxide dismutase activity was present in the heterocysts and vegetative cells of *Anabaena cylindrica*, but was always lower in the heterocysts. 2. No qualitative differences were found in the superoxide dismutase from the two cellular types. 3. Catalase activity was also present in both cellular types. 4. Most of the NADP reductase activity, as assayed with menadione or ferredoxin as electron acceptor, was localized within the heterocysts. 5. Studies on H₂ consumption showed that most of the hydrogenase activity was associated with the heterocysts. 6. The results are discussed in terms of the postulate that superoxide dismutase and catalase are involved in the protection of the proton-donating systems participating in N₂ fixation and H₂ metabolism of heterocysts.

A consequence of the sensitivity of nitrogenase and hydrogenase to O₂ is that most aerobic N₂-fixing organisms that have been examined, including blue-green algae (Cyanobacteria), do not fix N₂ or evolve H₂ optimally at atmospheric O₂ concentration (Postgate, 1971; Kondratieva & Gogotov, 1976; Haaker & Veeger, 1977). These processes occur ideally at low concentrations of O₂ in the gas phase (Postgate, 1971; Gogotov *et al.*, 1976).

Blue-green algae capable of fixing N₂ have a special problem because their photosynthetic metabolism results in O₂ evolution. A characteristic of some N₂-fixing blue-green algae such as *Anabaena cylindrica* is the presence of 'heterocysts', specialized cells that lack an O₂-evolving photosystem activity (Thomas, 1970). These specialized cells have been shown to contain a nitrogenase system, which can catalyse ATP-dependent production of H₂, in addition to containing a large part of the hydrogenase activity (Haystead *et al.*, 1970; Tel-Or & Packer, 1976). However, the nature of oxygen toxicity and the mechanism of protecting the oxygen-labile components are not understood.

Extensive studies over the past decade have clearly demonstrated that univalent reduction of O₂ does occur in biological systems (Bors *et al.*, 1974; Fridovich, 1975; Malmström *et al.*, 1975):

As the pK_a of HO₂[·] is 4.8, the superoxide anion radical (O₂^{·-}) is thus present under physiological

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conditions where it is capable of acting as an oxidizing agent as exemplified by the reaction:

or as a reducing agent as in the following reaction:

It is therefore essential that the superoxide anion radicals are maintained at concentrations compatible with biochemical processes. The enzyme superoxide dismutase (O₂^{·-}-O₂^{·-} oxidoreductase, EC 1.15.1.1) is thought to constitute the primary defence of cells against this potential toxicity by catalysing the following reaction:

H₂O₂ can be removed by enzymes such as catalase (H₂O₂-H₂O₂ oxidoreductase, EC 1.11.1.6), which catalyse the reaction:

It was decided to investigate the activities of superoxide dismutase and catalase *in vivo* in relation to O₂ concentration. The blue-green alga *Anabaena cylindrica* provides a useful model for such a study and the problem was investigated with reference to the protection of the proton-donating systems of N₂ fixation.

Materials and Methods

Isolated heterocysts (over 90% purity) and entire filaments of *Anabaena cylindrica* were a kind gift from

Dr. E. Tel-Or (Department of Biological Sciences, University of Dundee, Dundee, Scotland, U.K.), (see Tel-Or & Stewart, 1976). The chemicals used were of the highest quality commercially available and were obtained from BDH Chemicals (Poole, Dorset, U.K.) or Sigma (Kingston upon Thames, Surrey, U.K.).

Preparation of extracts

All procedures were carried out at 0–4°C unless otherwise stated. The cells were lysed by intermittent sonication in 50 mM-Tris/HCl/0.1 mM-EDTA, pH 7.8, with a Soniprobe (type 1130A; Dawe Instruments, Acton, London, U.K.). The vegetative cells required a total of 4 min at a power output of 1.5–2.0 A, whereas the heterocysts required 35 min at 5.0–5.5 A. During the sonication the temperature was kept below 5°C by an ice/NaCl bath. At regular intervals during the sonication the cells were examined under a phase-contrast microscope to ascertain the degree of intactness of the cells. The vegetative cells contaminating the heterocyst preparation were removed by washing after their disruption.

(NH₄)₂SO₄ fractionation

After sonication the homogenates were centrifuged at 40000g for 20 min. The supernatant was made to 40% (NH₄)₂SO₄ saturation by the addition of the solid, at 4°C. The pH was maintained at 7.8 by the addition of aq. NH₃ where necessary. The solution was then centrifuged at 40000g for 30 min and the supernatant made 90% saturated with (NH₄)₂SO₄ after leaving for 1 h. The solution was again centrifuged at 40000g for 30 min. The pellet was then redissolved in 5 mM-Tris/HCl, pH 7.8, and dialysed against this buffer until the conductivity of the extract was similar to the conductivity of 5 mM-Tris/HCl, pH 7.8.

Enzyme assays

Spectrophotometric assays were performed at 20°C. Superoxide dismutase was assayed by three different methods: the xanthine/xanthine oxidase system (Halliwell, 1975), the riboflavin/methionine system (Beauchamp & Fridovich, 1971) and the KO₂ method (Henry *et al.*, 1976). Nitro Blue Tetrazolium was used in each of the assay systems and the development of the blue colour caused by formazan production was followed at 560 nm. The results are expressed in units of activity as measured by the xanthine/xanthine oxidase system; 1 unit of activity is defined as that amount causing a 50% decrease in the rate of Nitro Blue Tetrazolium reduction under the conditions of the assay. Catalase was measured by a polarographic method as described by Rich *et al.* (1976); 1 unit is defined as that amount which

catalyses the breakdown of 1 μmol of H₂O₂/min at pH 7.0 and at 20°C at 12.5 mM-H₂O₂. Hydrogenase activity was assayed by the rate of H₂ consumption by using Methyl Viologen as proton acceptor or by H₂ evolution with sodium dithionite as the electron donor and Methyl Viologen as mediator. The reaction mixture (final volume 2 ml) in 15 ml vials contained 20 mM-sodium/potassium phosphate buffer, pH 7.0, and 5 mM-Methyl Viologen. After flushing with N₂, fresh sodium dithionite (10 mM) was added. The vials were incubated at 30°C and the reactions started by the addition of the preparations. H₂ consumption or production was determined by gas chromatography as described by Rao *et al.* (1976). The reductase was assayed by spectrophotometric measurements of NADPH oxidation at 340 nm with menadione under aerobic conditions after the addition of 0.05 ml of NAD(P)H-regenerating system (Hatchikian, 1970). The reduction of ferredoxin was carried out under anaerobic conditions (Yoch, 1973). Reaction mixture (2.0 ml) contained *Spirulina maxima* ferredoxin (0.14 mM), NADPH-regenerating system containing glucose 6-phosphate (10 μmol), NADP⁺ (1 μmol), glucose 6-phosphate dehydrogenase (10 μg), cell extract after (NH₄)₂SO₄ fractionation and 50 mM-glycine buffer, pH 9.0.

Electrophoresis

Polyacrylamide-gel electrophoresis was carried out as described by Davis (1964) in 10 or 7.5% polyacrylamide resolving gels with stacking gels of 2.5%. The buffer was 25 mM-Tris/HCl/0.192 M-glycine, pH 8.5. After electrophoresis at 3 mA/tube the gels were stained for superoxide dismutase activity by the method of Beauchamp & Fridovich (1971) or for peroxidase by the method of Smith *et al.* (1970).

Isoelectric focusing was carried out by the method of Gainer (1973); 5.5 and 7.5% gels were prepared with a final Ampholine concentration of up to 10% (v/v). Ampholine carrier ampholytes (LKB, South Croydon, Surrey, U.K.) in the pH ranges 3–5 and 4–6 were used. Isoelectric focusing was carried out from 6 to 16 h at 4°C at constant voltage with a maximum of 20 V/tube. At the termination of the experiment each gel was cut longitudinally, one half stained for superoxide dismutase and the other half cut into 0.5 cm sections and incubated for 5.0 h in 0.5 ml of CO₂-free water at 4°C. The pH of each solution was determined at the same temperature as the isoelectric focusing after this 5.0 h period and is taken to represent the pH of that portion of the gel.

Partial purification of superoxide dismutase

After (NH₄)₂SO₄ fractionation the extract was adjusted to 2.5% saturation with streptomycin

sulphate by the addition of a 25% solution; the mixture was gently stirred at 4°C for 30 min, then centrifuged at 40000g for 20 min. The supernatant containing the superoxide dismutase was extensively dialysed against 5 mM-Tris/HCl, pH 7.8. It was then adsorbed on a DEAE-cellulose (DE 52; Whatman, Maidstone, Kent, U.K.) column, washed with 50 mM-Tris/HCl, pH 7.8, then eluted with 3 column volumes of a linear gradient of 0–300 mM-KCl in 50 mM-Tris/HCl buffer, pH 7.8.

Additional measurements

Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. Chlorophyll *a* was determined by the method of Barr & Crane (1971).

Results

Pigment composition

Optical absorption spectra, in the visible region, of the 40000g supernatant (see the Materials and Methods section) from the two types of cellular homogenates showed that C-phycoerythrin, maximum absorption about 620 nm, was present in the vegetative cells only. The two cellular types were shown to have the same type of chlorophyll, absorption maximum 675–680 nm.

Superoxide dismutase and catalase

Table 1 shows that the activity of superoxide dismutase within the heterocysts is lower than that in the vegetative cells. In terms of both units/mg of soluble protein and units/mg of chlorophyll *a* the activity measured for the heterocysts preparations was less than 30% of the total detectable activity.

The catalase activity within the different cellular types appears more complex. In terms of units/mg of soluble protein about 50% of the total activity was present in the heterocysts. However, on a chlorophyll *a* basis the activity within the heterocysts was less than 30% of the total activity. The reason for this apparent anomaly is not known.

Electrophoretic analysis

After electrophoretic fractionation staining for superoxide dismutase revealed two closely adjacent bands of activity. An identical pattern for the vegetative cells and the heterocysts were obtained and both isoenzymes were insensitive to cyanide treatment (1 mM). Neither of these activity zones could be attributed to the presence of peroxidase.

Isoelectric focusing in polyacrylamide gels indicated the presence of only one activity band, which corresponded to a pH of 3.9 ± 0.1 and was taken to represent the isoelectric point of the protein.

The patterns obtained after normal polyacrylamide-gel electrophoresis and isoelectric focusing were similar when crude and partially purified extracts were examined.

In Table 2 the scheme used during the partial purification of the superoxide dismutase is presented. After the DEAE-cellulose chromatography the enzyme from the vegetative cells had a specific activity of 360 units/mg of protein, which represents a purification of 18-fold. From conductivity measurements the elution of the protein from the column required 80–100 mM-KCl in 50 mM-Tris/HCl buffer, pH 7.8, at 4°C. The superoxide dismutase from the heterocyst was purified 12-fold, giving a specific activity of 42.5 units/mg of protein.

In Table 3 the activity of enzymes systems involved in N₂ fixation and metabolism of H₂ are presented on a comparative basis. In terms of H₂ consumption

Table 1. Comparative study of superoxide dismutase and catalase in extracts from the vegetative cells and heterocysts of *Anabaena cylindrica*

The experimental details are given in the Materials and Methods section. The results are the means of three separate preparations $\pm$ S.D. Each preparation was assayed a minimum of three times. For definition of enzyme units refer to the text.

	Vegetative cells	Heterocysts
Superoxide dismutase	20.0 $\pm$ 2.0	5.3 $\pm$ 2.2
(units/mg of soluble protein; soluble protein after sonication)		
(units/10mg of chlorophyll <i>a</i>)	13.3 $\times 10^3 \pm 1 \times 10^3$	1.9 $\times 10^3 \pm 0.65 \times 10^3$
Catalase		
(units/mg of soluble protein; soluble protein after sonication)	0.164 $\pm$ 0.02	0.143 $\pm$ 0.025
(units/10mg of chlorophyll <i>a</i>)	101 $\pm$ 3	34.5 $\pm$ 8

Table 2. *Partial purification of superoxide dismutase from the vegetative cells and heterocysts of Anabaena cylindrica*

The results presented were from a typical preparation. See the text for experimental details and for definition of enzyme unit. Abbreviation: N.D., not determined.

Fractionation step	Preparations	Specific activity (units/mg of protein)	Purification factor
Crude extract	Vegetative cells	19.3	1
	Heterocysts	3.5	1
(NH ₄) ₂ SO ₄ fractionation (40–90% saturation)	Vegetative cells	95.0	5
	Heterocysts	42.5	12
Ion-exchange chromatography	Vegetative cells	360	19
	Heterocysts	N.D.	—

Table 3. *Comparative activity of certain enzymes involved in N₂-fixation in the vegetative cells and heterocysts of Anabaena cylindrica*

Details of the experiments are given in the Materials and Methods section. The results were from three preparations and are expressed as percentage of the total activity (on a protein basis).

Reactions	Activity (%)	
	Vegetative cells	Heterocysts
H ₂ consumption	7–15	85–93
NAD(P) reductase: NADPH + menadione	0–38	62–100
NADPH-regenerating system + ferredoxin	10–20	80–90

85–93% of the hydrogenase activity is localized within the heterocysts (H₂ production was not observed in either extract). Reductase activity was only observable with NADPH as the proton donor; no activity with either preparation was observed with NADH. Firstly, with a NADPH + menadione system the reductase activity present in the heterocyst preparation was considerably higher than that in the vegetative cells, between 62 and 100% of the activity being present in the heterocyst (range depends on the preparation). Secondly, with ferredoxin and a NADPH-regenerating system 80–90% of the reductase activity was detected within the heterocyst preparation.

Discussion

The lack of C-phycoerythrin in the heterocyst preparation indicates that Photosystem II is absent (see Donze *et al.*, 1972); thus we expect that there are

different O₂ concentrations within the different cellular types. In addition the absence of the C-phycoerythrin from our heterocyst preparations indicates that there was no contamination from vegetative cells. The activity of superoxide dismutase within the heterocyst is appreciably lower than that in the vegetative cells, and this may be correlated with their different O₂ content. It has been reported that although 'young' N₂-fixing heterocysts lack the C-phycoerythrin, old heterocysts can regain their O₂-evolving Photosystem-II components (Thomas & David, 1972).

The presence of superoxide dismutase within the heterocysts of *Anabaena cylindrica* was not surprising, in view of the fact that this enzyme has previously been reported to occur within cells sensitive to low O₂ concentration. In fact Hewitt & Morris (1975) and Lumsden & Hall (1975) reported its occurrence in obligate anaerobes such as *Chlorobium thiosulfatophilum*, *Clostridium perfringens* and *Chromatium D*. The lower activity of superoxide dismutase within the heterocysts than in the vegetative cells is in agreement with earlier reports correlating the activity of this enzyme in a number of organisms and the ambient O₂ concentration (Fridovich, 1975).

The question as to whether the heterocysts are anaerobic has not been resolved. The occurrence of superoxide dismutase and catalase within the heterocysts appears to offer the necessary protection against oxygen toxicity. Whether oxygen toxicity results from the re-oxidation of certain reduced components or from a direct attack by activated species such as the superoxide anion on labile systems is not known.

From the electrophoretic analysis no qualitative changes in the superoxide dismutases were observable in extracts obtained from the different cell types. Whether the different activities of superoxide dismutase detected represent a systematic breakdown of pre-existing enzyme or an inability to synthesize additional enzyme within the heterocyst is not known.

The absence of a cyanide-sensitive Cu/Zn-containing superoxide dismutase from *Anabaena cylindrica* is in agreement with previous reports concerning the absence of this type of dismutase from prokaryotes (Asada *et al.*, 1976; Lumsden & Hall, 1975). It is interesting that although two achromatic zones were observed after normal polyacrylamide-gel electrophoresis, only one was observed by using isoelectric focusing in polyacrylamide gels. The possibility that they represent components that differ in size cannot be dismissed; they may, however, represent distinct isoenzymes with the same isoelectric point.

Our results on the distribution of hydrogenase and ferredoxin or menadione–NADP reductase activity showed that the major part of these enzymes were localized in the heterocysts. These data are in agreement with results relating to the localization of nitrogenase and hydrogenase within the heterocysts

of *Anabaena cylindrica* by Tel-Or & Packer (1976). However, in our preparations both heterocysts and vegetative cells lacked the capability of H₂ evolution, although they showed good H₂-uptake activity. This may be correlated with inactivation of one of the components of H₂-evolving systems during extraction as anaerobic conditions were not used.

It is noteworthy that reduction of menadione in these extracts occurred only in the presence of NADPH (but no NADH), a reductant which is characteristic of plant-type photosynthesis.

In summary it therefore appears likely that the activities of superoxide dismutase and possibly catalase are dependent on the oxygen status within the heterocysts and vegetative cells of *Anabaena cylindrica*. These results are consistent with the premise that the physiological role of these enzymes is in limiting oxidant injury. Whether such injury accounts for the apparent sensitivity of the hydrogenase and nitrogenase systems to O₂ is not known at present.

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References

- Asada, K., Kanematsu, S., Takahashi, M. & Kono, Y. (1976) in *Iron and Copper Proteins* (Yasunobu, K. T., Mower, H. F. & Hayaishi, O., eds.), pp. 551–564, Plenum Press, New York and London
- Barr, R. & Crane, F. L. (1971) *Methods Enzymol.* **23**, 372–408
- Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287
- Bors, W., Saran, M., Lengfelder, E., Spöttl, R. & Michel, C. (1974) *Curr. Top. Radiat. Res. Q.* **9**, 247–309
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 407–427
- Donze, M., Haneman, J. & Schiereck, P. (1972) *Biochim. Biophys. Acta* **256**, 157–161
- Fridovich, I. (1975) *Adv. Enzymol.* **41**, 35–97
- Gainer, H. (1973) *Anal. Biochem.* **51**, 646–650
- Gogotov, I. N., Kosijak, A. V. & Krupenko, A. N. (1976) *Mikrobiologia* **45**, 941–945
- Haaker, H. & Veeger, C. (1977) *Eur. J. Biochem.* **77**, 1–10
- Halliwell, B. (1975) *FEBS Lett.* **56**, 34–38
- Hatchikian, E. C. (1970) *Biochim. Biophys. Acta* **212**, 353–355
- Haystead, A., Robinson, R. & Stewart, W. D. P. (1970) *Arch. Mikrobiol.* **74**, 235–243
- Henry, L. E. A., Halliwell, B. & Hall, D. O. (1976) *FEBS Lett.* **66**, 303–306
- Hewitt, J. & Morris, J. G. (1975) *FEBS Lett.* **50**, 315–318
- Kondratieva, E. N. & Gogotov, I. N. (1976) *Izv. Akad. Nauk U.S.S.R. Biol. Ser.* **1**, 69–86
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lumsden, J. & Hall, D. O. (1975) *Nature (London)* **257**, 670–672
- Malmström, B. G., Andreasson, L. E. & Reinhammar, B. (1975) *Enzymes 3rd Ed.* **12**, 533–557
- Postgate, J. R. (1971) in *The Chemistry and Biochemistry of Nitrogen Fixation* (Postgate, J. R., ed.), pp. 161–190, Plenum Press, London and New York
- Rao, K. K., Rosa, L. & Hall, D. O. (1976) *Biochem. Biophys. Res. Commun.* **68**, 21–28
- Rich, P. R., Boveris, A., Bonner, W. D., Jr. & Moore, A. L. (1976) *Biochem. Biophys. Res. Commun.* **71**, 695–703
- Smith, H. H., Hamill, D. E., Weaver, E. A. & Thompson, K. H. (1970) *J. Hered.* **61**, 203–212
- Tel-Or, E. & Packer, L. (1976) in *Proc. Int. Symp. Photosynthetic Prokaryotes 2nd* (Codd, G. A. & Stewart, W. D. P., ed.), pp. 140–142, Elsevier/North Holland, Amsterdam
- Tel-Or, E. & Stewart, W. D. P. (1976) *Biochim. Biophys. Acta* **423**, 189–195
- Thomas, J. (1970) *Nature (London)* **228**, 181–183
- Thomas, J. & David, K. A. V. (1972) *Nature (London) New Biol.* **238**, 219–221
- Yoch, D. C. (1973) *J. Bacteriol.* **116**, 384–391