Immunocytochemical Localization of Thioredoxins in the Cyanobacteria *Anabaena cylindrica* and *Anabaena variabilis*

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(Received 25 March 1985; revised 2 July 1985)

Colloidal gold conjugated goat anti-rabbit IgG and specific antisera raised against purified thioredoxins from *Anabaena cylindrica* and *Anabaena variabilis* have been used to determine the localization of thioredoxin in whole filaments of *A. cylindrica* and *A. variabilis*. Vegetative cells of *A. cylindrica* were labelled differently when tested with either of the two anti-thioredoxin sera. The anti-*A. cylindrica* thioredoxin serum reacted only with *A. cylindrica* thioredoxin which was located predominantly in the nucleoplasm in vegetative cells; it showed no association with the chromatoplasm. Anti-*A. variabilis* thioredoxin serum labelled vegetative cells of *A. variabilis*, particularly in the vicinity of the thylakoid membranes and also labelled *A. cylindrica* in the vicinity of the thylakoid membranes, indicating that a second thioredoxin or thioredoxin-like protein was present in this cyanobacterium. Heterocysts of *A. cylindrica* and *A. variabilis* showed substantially less labelling than vegetative cells when tested with both antisera.

INTRODUCTION

Heterocysts, which usually account for 5 to 8% of the cells present in N₂-fixing heterocystous cyanobacteria, are the sites of N₂-fixation under aerobic conditions (see Stewart, 1980). There is controversy as to whether they are the sole sites of nitrogenase activity under strictly anaerobic conditions (Fleming & Haselkorn, 1974; Murry *et al.*, 1984). Heterocysts are also the main site of assimilation, via glutamine synthetase, of newly fixed NH₄⁺ (Dharmawardene *et al.*, 1973; Thomas *et al.*, 1977; see also Stewart, 1980). However, heterocysts lack photosystem 2 activity (see Tel-Or & Stewart, 1977; Ho & Krogmann, 1982) and do not synthesize ribulose-1,5-bisphosphate carboxylase/oxygenase, the key carboxylating enzyme of the Calvin cycle (see Cossar *et al.*, 1985). The oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase is present and active in the light and dark, in contrast to vegetative cells where it is fully active only in the dark (Apte *et al.*, 1978).

To date, information on the distribution of proteins between vegetative cells and heterocysts (Lex & Carr, 1974; Winkenbach & Wolk, 1973; Apte *et al.*, 1978; Neuer *et al.*, 1983) has depended on the use of techniques of heterocyst isolation which involve selective disruption of vegetative cells. The main drawbacks of such techniques are that the heterocysts are at various stages of maturity and it is difficult to assess their 'intactness' and the degree of vegetative cell contamination. Here we have used an immunocytochemical technique which uses specific antisera and colloidal gold in order to determine the intracellular location of thioredoxins (see Horisberger, 1983) in vegetative cells and heterocysts of *Anabaena cylindrica* and *Anabaena variabilis*.

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METHODS

Organisms and growth conditions. Axenic cultures of *Anabaena cylindrica* Lemm. (CCAP 1403/2a) and *Anabaena variabilis* Kütz. (ATCC 29413) were grown in nitrogen-free BG-11 medium (Stanier *et al.*, 1971) in 10-litre continuous cultures at a dilution rate of 0.005 h^{-1} at 25°C , at a photon flux density of $120\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, incident at the surface of the vessel, and gassed with air at a rate of 1 litre min^{-1} .

Preparation of antisera. Antisera against purified thioredoxins from *A. cylindrica* and *A. variabilis* were raised in New Zealand White rabbits as described previously (Ip *et al.*, 1984). On the basis of amino acid sequence data, both thioredoxins were structurally similar to thioredoxin *m* rather than to thioredoxin *f* of chloroplasts (Tsugita *et al.*, 1983).

Immunocytochemical labelling studies. The method used was a modification of that described by Beesley *et al.* (1984). Cyanobacteria were harvested by centrifugation at $12000g$ for 1 min, resuspended in 2.5% (v/v) glutaraldehyde in 0.1 M-potassium phosphate buffer (pH 7.2) and incubated for 2 h at 20°C . Excess glutaraldehyde was removed by washing three times for 15 min each in 0.1 M-potassium phosphate buffer (pH 7.2). The samples were then dehydrated in a graded ethanol series and embedded in LR 'white' resin (London Resin Co., Basingstoke, UK). Thin sections were cut using a Reichert OMU3 ultramicrotome and floated on to 300 mesh gold grids. Except where indicated in the figure legend, sections were then incubated for 2 h at room temperature in a 2% (v/v) dilution of the specific antiserum (concentrations of undiluted antisera were 10.0 mg ml^{-1} for serum Ac and 18.5 mg ml^{-1} for serum Av) in 50 mM-Tris/HCl buffer (pH 7.4) containing 1% (v/v) Tween 20, 1% (w/v) bovine serum albumin and 0.2% (w/v) gelatin, washed for 20 min in water containing 0.02% (w/v) KCl, and incubated for 1 h on drops of 5 nm or 20 nm colloidal gold (approximately 10^{12} particles ml^{-1}) conjugated to goat anti-rabbit IgG (GAR G5 and GAR G20, respectively) (Janssen Pharmaceutical, Beerse, Belgium). The sections were then washed for 20 min in water containing 0.02% (w/v) KCl, dried in air and stained for 20 h in osmium tetroxide vapour at 20°C . For control treatments, pre-immune rabbit serum was used instead of the specific antiserum. Sections were examined using an Associated Electrical Industries EM801 transmission electron microscope.

RESULTS

Localization of thioredoxin in A. variabilis

Vegetative cells of *A. variabilis* were extensively labelled, particularly in the vicinity of the thylakoids, when treated with antiserum against pure *A. variabilis* thioredoxin (serum Av) (Fig. 1*a-c*). The labelling of heterocysts was always lower than that of adjacent vegetative cells (Fig. 1*b, c*) and was sometimes negligible (Fig. 1*c*). This labelling was affected by the concentration of antiserum used and by the length of time during which the samples were exposed to the antiserum, and it varied also with the morphological state of the heterocyst, decreasing with heterocyst age.

Antiserum against pure *A. cylindrica* thioredoxin (serum Ac) did not react with cell-free extracts of *A. variabilis* on rocket immunoelectrophoresis (Ip *et al.*, 1984). Labelling of *A. variabilis* cells with this antiserum was similar to that obtained with pre-immune serum (data not shown), confirming the lack of cross-reactivity between *A. variabilis* thioredoxin and serum Ac.

Localization of thioredoxin in A. cylindrica

Vegetative cells of *A. cylindrica* were extensively labelled when treated with serum Ac (Fig. 2) particularly in the nucleoplasmic region (Fig. 2*a-c, f*). Again, the labelling in the heterocysts (Fig. 2*d, e*) decreased with heterocyst age and labelling of some mature heterocysts (Fig. 2*d*) was no different from that obtained with pre-immune serum (Fig. 3*h*). Labelling of developing heterocysts (Fig. 2*e*) was similar to that of vegetative cells (Fig. 2*a-c, f*).

Fig. 3 shows the results obtained when *A. cylindrica* cells were treated with serum Av. In contrast to serum Ac, labelling was almost exclusively associated with the chromatoplasm (Fig. 3*a-d*). Again, however, the labelling of heterocysts (Fig. 3*f, g*) was always lower than that of vegetative cells, and was higher than that obtained with pre-immune serum (Fig. 3*e, h*). Under the labelling conditions used in these experiments, there was extensive binding of antisera to the heterocyst envelope of *A. cylindrica*. This was attributed to non-specific binding of immunoglobulins since labelling was equally extensive whether specific antisera (Fig. 2*d, e*; Fig. 3*f, g*) or pre-immune sera (Fig. 3*h*) were used.

Fig. 1. Localization of thioredoxin in *Anabaena variabilis*. Sections were labelled with GAR G20 following treatment with anti-*A. variabilis* thioredoxin serum (2%, v/v) for 2 h at room temperature (a, b), at a higher concentration (10%, v/v) for 18 h at 4 °C (c) or with pre-immune serum (2%, v/v) (d). V, vegetative cell; H, heterocyst; bars, 0.5 μ m.

DISCUSSION

We have purified a single, soluble thioredoxin from the cyanobacteria *A. cylindrica* and *A. variabilis*. Each of these thioredoxins was identified on the basis of its ability to activate several enzymes, including NADP⁺-dependent malate dehydrogenase and fructose-1,6-bisphosphatase, and to deactivate glucose-6-phosphate dehydrogenase (see Buchanan, 1980; Cossar *et al.*, 1984). On the basis of their partial amino acid sequences, the thioredoxins from *A. cylindrica* and *A. variabilis* (Ip *et al.*, 1984; A. J. Darling, P. Rowell & W. D. P. Stewart, unpublished results) are structurally similar to other bacterial thioredoxins (Holmgren, 1968; Meng & Hogenkamp, 1981) and to chloroplast thioredoxin *m* (Tsugita *et al.*, 1983), but they differ in some other respects including their antigenicity (our unpublished results).

The labelling patterns of *A. cylindrica* with the two antisera (Ac and Av) strongly suggest the presence of two different thioredoxins or thioredoxin-like proteins in this organism as in certain other cyanobacteria (Schmidt, 1980; Yee *et al.*, 1981; Whittaker & Gleason, 1984) and that these may have different intracellular and intercellular locations. The major thioredoxin (see Ip *et al.*, 1984), with which serum Ac reacts, is a nucleoplasmic protein which occurs in vegetative cells but which may be absent from, or present at very much reduced levels in mature heterocysts. It resembles, in some respects, thioredoxin of *Escherichia coli* which occurs mainly in association with the plasmalemma/periplasmic space and in the nuclear region (Lunn & Pigiet, 1982;

Fig. 2. Localization of thioredoxin in *Anabaena cylindrica* using anti-*A. cylindrica* thioredoxin serum (a-f). Sections were labelled with GAR G20 following pre-treatment with the anti-*A. cylindrica* thioredoxin serum (2%, v/v) for 2 h at room temperature. V, vegetative cell; H, heterocyst; N, nucleoplasm; C, chromatoplasm; bars, 0.5 μ m.

Nygren *et al.*, 1982). The other, which reacts with serum Av, is associated with the chromatoplasm of vegetative cells and is present at reduced levels in heterocysts. Serum Av does not react with purified thioredoxin from *A. cylindrica* (Ip *et al.*, 1984) on rocket immunoelectrophoresis, but we have recently also purified a minor thioredoxin-like protein which appears to correspond to the protein with which serum Av reacts in the chromatoplasm of both *A. cylindrica* and *A. variabilis*. A thylakoid membrane-associated thioredoxin has previously been reported from chloroplasts (Ashton *et al.*, 1980).

Fig. 3. Localization of thioredoxin in *Anabaena cylindrica* using anti-*A. variabilis* thioredoxin serum. Sections were labelled with GAR G20 (a-c, e-h) or GAR G5 (d) following treatment with anti-*A. variabilis* thioredoxin serum (2%, v/v) (a-d, f, g) or pre-immune serum (2%, v/v) (e, h). V, vegetative cell; H, heterocyst; N, nucleoplasm; C, chromatoplasm; bars, 0.5 μ m.

The true physiological functions of cyanobacterial thioredoxins are incompletely understood at present. It is possible that the chromatoplasm-associated thioredoxin(s) are involved in the modulation of the activities of Calvin cycle enzymes (see Buchanan, 1980; Yee *et al.*, 1981), the coupling factor of photophosphorylation (Mills *et al.*, 1981), and of glucose-6-phosphate dehydrogenase (Cossar *et al.*, 1984). The soluble thioredoxin of *A. cylindrica*, on the other hand, which is structurally similar to *E. coli* thioredoxin (Holmgren, 1968; Ip *et al.*, 1984), is located mainly in the nucleoplasm and may be involved in DNA synthesis as in *E. coli* where it may function in ribonucleotide reduction (Laurent *et al.*, 1964). Thioredoxin is also required for the functioning of DNA polymerase of bacteriophage T₇ (Mark & Richardson, 1976; Mark *et al.*, 1977) and possibly in plasmid replication in *E. coli* (Lunn *et al.*, 1984). The apparent absence of this thioredoxin from heterocysts may be associated with the inability of mature heterocysts to de-differentiate (Wilcox *et al.*, 1973).

We have demonstrated previously that reduced thioredoxin can deactivate glucose-6-phosphate dehydrogenase of *A. variabilis* (Cossar *et al.*, 1984; see also Udvardy *et al.*, 1984). This may be important in vegetative cells for the inhibition of the oxidative pentose phosphate pathway in the light (Pelroy *et al.*, 1972; Duggan & Anderson, 1975; Apte *et al.*, 1978). We have further postulated (Stewart *et al.*, 1985) that the decreased level of thioredoxins in heterocysts of *A. cylindrica* and *A. variabilis* may be related to the high activity of glucose-6-phosphate dehydrogenase in such cells, thus allowing reductant for nitrogenase to be provided by the oxidative pentose phosphate pathway both in the light and dark (Stewart *et al.*, 1985). Further evidence, for or against this hypothesis, necessitates an examination for both thioredoxin and nitrogenase proteins in single heterocysts. Studies are in progress to determine more precisely the correlation of thioredoxin levels with the stages of differentiation of heterocysts.

We thank the Agricultural and Food Research Council, the Science and Engineering Research Council, the Natural Environment Research Council and the Royal Society for financial support. We are indebted to Dr Julian Beesley, Wellcome Research Co., Beckenham, Kent, for assisting with the immunocytochemical labelling method. We also thank Mrs E. M. Lloyd-Davies for help with the electron microscopy.

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