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Light-induced transitions in the redox state of thioredoxin in the N_2-fixing cyanobacterium *Anabaena cylindrica*

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The redox state of thioredoxin in intact filaments of the N_2-fixing cyanobacterium *Anabaena cylindrica* was investigated, using a method which involves carboxymethylation of the cysteine residues of reduced thioredoxin to prevent oxidation and immunoelectrophoretic determination of the reduced (carboxymethylated) and oxidized forms of the protein. In filaments grown in continuous light (55 μmol photons per m^2 per s) approx. 70% of the thioredoxin was present in the reduced form. On transfer from light to dark the level of reduced thioredoxin decreased over 2–3 h, to about 10% of the total. On re-illumination, the proportion of reduced thioredoxin increased rapidly to the original steady state (light) level. Such changes in the redox state of thioredoxin are consistent with a role in the light-dependent regulation of metabolism in this photosynthetic prokaryote.

Introduction

Thioredoxins are acidic, heat-stable, dithiol-containing proteins of molecular mass of about 12 kDa which may be involved in the light-dependent modulation of key enzymes of several metabolic pathways in higher plants, algae and cyanobacteria (see Refs. 1–4). Thioredoxin may also serve as the hydrogen donor for processes such as ribonucleotide reduction [4,5]. Thioredoxins can be reduced in vivo by NADPH-dependent or ferredoxin-dependent thioredoxin reductases [6–8]. Little is known about the redox state of thioredoxin in vivo, with limited information being available only for *Escherichia coli* [9] and thioredoxin *m* of isolated chloroplasts [10]. It is clearly important for an understanding of the physiological functions of thioredoxins, to be able to determine under what conditions and to what extent they are oxidized and reduced in vivo. Here, we describe a method for the estimation of oxidized and reduced thioredoxins and describe its use to examine the influence of light on the redox state of thioredoxin in the N_2-fixing cyanobacterium *Anabaena cylindrica*.

Materials and Methods

Organism and growth conditions. Anabaena cylindrica Lemm (CCAP 1403/2a) was grown axenically in BG-11 medium [11] in 0.1 l or 10 l batch cultures, aerated at 1 l per min, at 25°C and at a photon flux density of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ incident at the surface of the vessel. All material was from exponential phase cultures (2.5 μg Chl per ml culture).

Abbreviations: Chl, chlorophyll; CTAB, cetyltrimethylammonium bromide

Preparation of cell-free extracts, purification of thioredoxin, polyacrylamide gel electrophoresis and immunoelectrophoresis. These were carried out as before [3] with minor modifications of the immunoelectrophoretic method as detailed in the legend to Fig. 2.

Carboxymethylation of thioredoxin. Thioredoxin was incubated with 2.5 mM dithiothreitol in 50 mM Tris-HCl buffer (pH 7.5) for 10 min at 20°C or with 2.5 mM dithiothreitol plus 6 M guanidium hydrochloride (to denature the protein) in 0.5 M Tris-HCl buffer (pH 8.5) under N_2 for 1 h at 20°C. Iodoacetate was then added to 15 mM and incubation was continued for a further 20 min [10,12]. The carboxymethylated thioredoxin was then dialysed against 50 mM Tris-HCl buffer (pH 7.5) before electrophoresis.

Results

Electrophoretic separation of the oxidized and reduced forms of thioredoxin

Oxidized thioredoxin purified from *Anabaena cylindrica* produced a single band on polyacrylamide gel electrophoresis (Fig. 1, lane 1). After treatment with 2.5 mM dithiothreitol, a second band, which was attributed to the presence of reduced thioredoxin [13] was also observed (Fig. 1, lane 3). The fact that only partial conversion to the reduced form was observed was probably due to re-oxidation during electrophoresis [13]. It is, therefore, possible to distinguish between the oxidized and reduced forms of thioredoxin on the basis of their different electrophoretic mobilities, but it is necessary to prevent reoxidation.

This can be achieved by modification of the two thiol groups in the reduced form of the protein. Treatment of dithiothreitol-reduced thioredoxin with iodoacetate, to carboxymethylate the sulphydryl groups, resulted in the formation of two new protein species on electrophoresis (Fig. 1, lane 4). By analogy with *E. coli* thioredoxin [9,12], the major and minor bands were attributed to the mono- and dicarboxymethylated forms of thioredoxin, respectively. Treatment with iodoacetate had no effect on oxidized thioredoxin in the absence (Fig. 1, lane 2) or presence (Fig. 1, lane 5) of 6 M guanidium hydrochloride, and only the band attributed to the dicarboxymethylated form was obtained when the protein was treated with 2.5 mM dithiothreitol in the presence of guanidium hydrochloride prior to iodoacetate treatment (Fig. 1, lane 6). Studies on the reactivities of cysteine residues 32 and 35 of *E. coli* thioredoxin [12] have shown that, on treatment of the reduced form of the protein with iodoacetate at pH 7.4, only the monocarboxymethylated form (at cysteine 32) was formed, due to the different reactivities of the two cysteine residues in the native protein. However, the two cysteine residues of the denatured protein showed a similar reactivity.

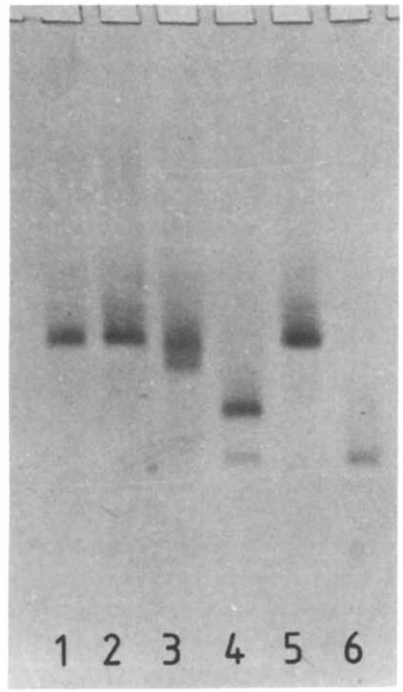

Fig. 1. Polyacrylamide gel electrophoresis of purified *A. cylindrica* thioredoxin on a 15 cm×15 cm gel containing 7.5% (w/v) acrylamide and 0.27% (w/v) *N,N'*-methylenebisacrylamide in 0.1 M Tris-glycine buffer (pH 8.9). Lane 1, oxidized thioredoxin (5 μg); lane 2, oxidized thioredoxin (5 μg) after treatment with 15 mM iodoacetate; lane 3, thioredoxin (5 μg) incubated for 10 min with 2.5 mM dithiothreitol, lane 4, thioredoxin (5 μg) after incubating for 10 min with 2.5 mM dithiothreitol, then with 15 mM iodoacetate; lane 5, oxidized thioredoxin (5 μg) incubated with 6 M guanidium hydrochloride; lane 6, thioredoxin (1 μg) incubated with 6 M guanidium hydrochloride plus 2.5 mM dithiothreitol then with 15 mM iodoacetate (for details, see Materials and Methods).

Quantitative determination of oxidized and reduced thioredoxin by immunoelectrophoresis

Antiserum against *A. cylindrica* thioredoxin [3] was used in 1-dimensional rocket immunoelectrophoresis to quantify the oxidized and reduced (monocarboxymethylated and dicarboxymethylated) forms of thioredoxin. In each case the rocket area was directly proportional to the amount of thioredoxin in the range 30–300 ng (0.63 $mm^2 \cdot ng^{-1}$ thioredoxin, using a 400-fold dilution of antiserum) and carboxymethylation did not affect the relationship between rocket area and amount of thioredoxin.

In order to determine the quantities of oxidized and carboxymethylated thioredoxin in mixtures such as cell-free extracts, 2-dimensional immunoelectrophoresis was used. Using purified thioredoxin, the oxidized form (Fig. 2a) had a much lower mobility than the reduced forms

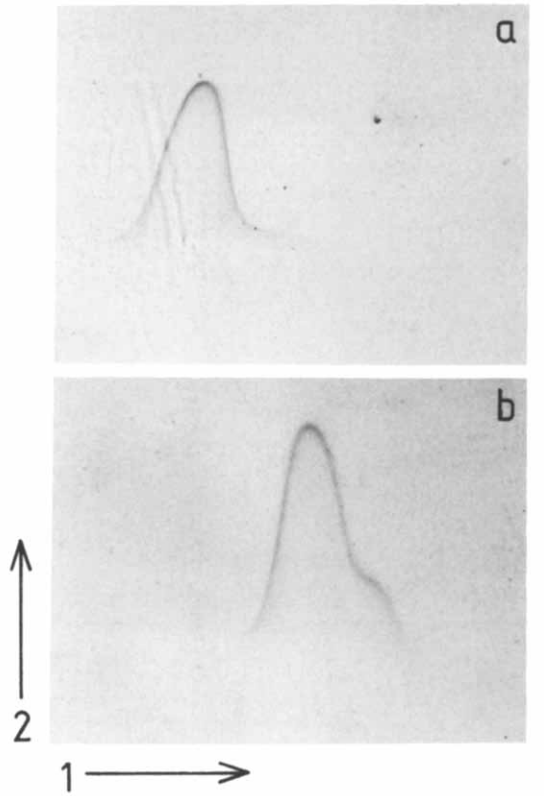

Fig. 2. Two-dimensional immunoelectrophoretic analysis of purified thioredoxin. (a) oxidized thioredoxin (115 ng); (b) thioredoxin (180 ng) after incubating for 10 min with 2.5 mM dithiothreitol, then with 15 mM iodoacetate. Arrows 1 and 2 indicate directions of migration in first and second dimensions of electrophoresis, respectively. Gels (12 cm × 5 cm) contained (second dimension) 25 μg anti-*A. cylindrica* thioredoxin serum (0.25%, v/v) and were used to allow complete separation of thioredoxin in the first dimension (12 cm). Electrophoresis was at 600 V for 30 min in the first dimension and at 50 V for 18 h in the second dimension.

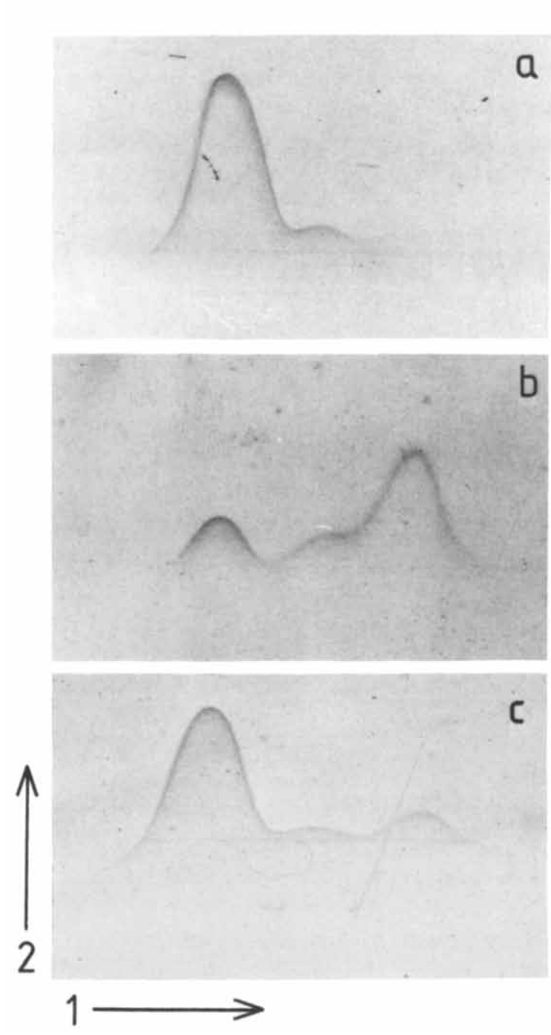

Fig. 3. Two-dimensional immunoelectrophoretic analysis of thioredoxin in cell-free extracts of N_2-fixing *Anabaena cylindrica*. (a) cell-free extract (52 μg protein) of a culture grown in continuous light; (b) cell-free extract (56 μg protein) of filaments from a culture grown in continuous light and incubated with 0.02% (w/v) CTAB plus 15 mM iodoacetate for 15 min prior to preparation of the cell-free extract; (c) cell-free extract (45 μg protein) of filaments from a culture grown in continuous light, then incubated in darkness for 2 h prior to treatment with CTAB plus iodoacetate as in (b). Conditions of electrophoresis were as described in the legend to Figure 2.

(mono- and dicarboxymethylated) (Fig. 2b) which, having similar electrophoretic mobilities, formed a pair of fused rockets.

Determination of the in vivo redox state of thioredoxin in A. cylindrica

When freshly prepared cell-free extracts of N_2-fixing light-grown *A. cylindrica* were analysed by 2-dimensional immunoelectrophoresis the major peak obtained corresponded to oxidized thioredoxin with only a small proportion of the protein being in the reduced form (Fig. 3a). In contrast, when such cultures were made permeable to small molecules by treatment with 0.02% (w/v) cetyltrimethylammonium bromide (CTAB) in the presence of 15 mM iodoacetate at pH 7.5 for 15 min before preparation of cell-free extracts, 70 ± 4% of the thioredoxin was present in the reduced from (Fig. 3b). Reduced thioredoxin was, in this case, present mainly in the dicarboxymethylated form (Fig. 3b) in contrast to purified dithiothreitol-reduced thioredoxin (Fig. 2b) which occurred mainly in the monocarboxymethylated form. The reason for this difference remains to be determined. When light-grown cultures were incubated in darkness prior to such treatment (Fig. 3c) most of the thioredoxin was in the oxidized form. Identical results (data not shown) were obtained for cultures grown in BG-11 medium supplemented with NH_4Cl or $NaNO_3$ indicating that the redox state of thioredoxin is probably not dependent on the inorganic nitrogen source available for growth.

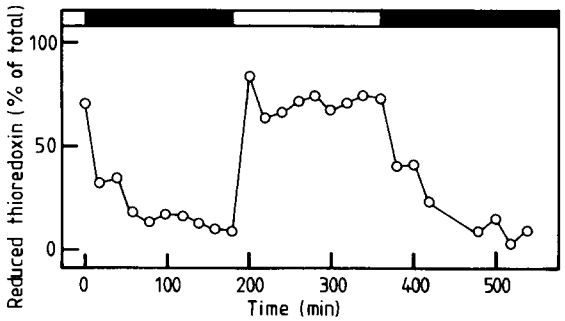

Fig. 4. The effects of light-dark transitions on the redox state of thioredoxin in N_2-fixing *Anabaena cylindrica*. The culture was incubated in the light or in darkness as indicated by the light and dark bars, respectively. Aliquots of culture, taken at timed intervals, were treated as described in the legend to Fig. 3b and c and Materials and Methods. Following 2-dimensional immunoelectrophoresis 'rocket' areas were measured and the proportions of reduced and oxidized thioredoxin calculated.

The effect of light-dark transitions on the redox state of thioredoxin in A. cylindrica

A culture grown in continuous light to mid-exponential phase was subjected to alternating periods of light and dark and the levels of oxidized and reduced thioredoxin were determined as described above at timed intervals. As Fig. 4 shows, thioredoxin was initially 70% reduced in the light-grown culture. Following transfer to darkness the proportion of reduced thioredoxin decreased to 30% after 40 min and to 10% after 3 h. On re-illumination, the proportion of reduced thioredoxin increased to over 80% within 20 min then decreased slightly and remained at about 70% over the subsequent 2.5 h period. When the culture was transferred from light to dark again, the proportion of thioredoxin in the reduced form decreased in a similar way to that observed in the first dark period.

Discussion

In a previous paper, we described the purification and characterization of thioredoxin from *A. cylindrica* [3]. This thioredoxin, which is structurally similar to thioredoxin *m* of chloroplasts [14], was found to be located mainly in the nucleoplasm of the vegetative cells of *A. cylindrica*, but showed no association with the thylakoid membranes [15]. Cyanobacteria possess a ferredoxin-dependent thioredoxin reductase [7,8] which was presumably responsible for the light-dependent reduction of thioredoxin observed in the experiments described in this paper and therefore reduction would require the transfer of reducing potential from the thylakoid membranes to the nucleoplasm. The oxidation of thioredoxin, which occurred on transfer of cultures to darkness, was relatively slow ($t_{1/2} \approx 30$ min). It is not clear whether cyanobacteria possess a specific mechanism for the oxidation of thioredoxin or whether this is mainly due to oxidation as a result of thioredoxin-dependent metabolic processes and to autooxidation. There is, however, evidence that thioredoxins may be oxidized by ferredoxin-NADP

oxidoreductase [16] or by a cysteine oxidase identified in the cyanobacterium *Synechococcus* 6301 [17].

Our data on the location of this thioredoxin *m* in *A. cylindrica* [15] suggest that it does not function in the activation of enzymes of the Calvin cycle or, indeed, of enzymes which are located in the chromatoplasm [18]. However, it has been shown recently that cyanobacteria have a second thioredoxin, similar to chloroplast thioredoxin *f* [4], which may function in this way, and it is possible that this corresponds to the minor thioredoxin-like protein which was shown, in our immunocytochemical studies [15], to be located in the chromatoplasm of *A. cylindrica*.

The location of a thioredoxin *m* [15] in *A. cylindrica* and its light-dependent reduction are consistent with a role in, for example, DNA replication, as the hydrogen donor for ribonucleotide reductase [4,5,19]. The activity of this enzyme was shown to be minimal in dark-treated carbon-starved cells of *Anabaena* 7119, to increase upon illumination and, thereafter to increase transiently in every division cycle and to correlate positively with increases in cellular DNA content [19].

Acknowledgements

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