Thioredoxin as a Modulator of Glucose-6-phosphate Dehydrogenase in a N_2 -Fixing Cyanobacterium

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(Received 22 August 1983)

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme involved in fixed carbon dissimilation in photosynthetic micro-organisms; in heterocystous cyanobacteria it may also be implicated in the supply of reductant to nitrogenase. In crude cell-free extracts of the N_2 -fixing cyanobacterium *Anabaena variabilis* G6PDH activity was reversibly deactivated by the thiol agent dithiothreitol in the presence of a low molecular weight protein (12000 mol. wt). Glucose 6-phosphate reversed deactivation when added at high concentration, or prevented deactivation if added with the thiol. $NADP^+$, which, like glucose 6-phosphate, is a G6PDH substrate, also deactivated the enzyme; deactivation was reversed or prevented by adding glucose 6-phosphate or glutamine. Purified thioredoxin from *Anabaena cylindrica*, at very low concentrations (2 nM), deactivated purified G6PDH in a manner identical to that observed when crude extracts were used in the presence of dithiothreitol. Glutathione did not affect the enzyme.

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes which use water as source of reductant and fix CO_2 via the Calvin cycle with ribulose-1,5-bisphosphate carboxylase serving as the key carboxylating enzyme, as in higher plants. Fixed carbon dissimilation is mainly via the oxidative pentose phosphate pathway (Pelroy & Bassham, 1972) with glucose-6-phosphate dehydrogenase (G6PDH) being the first enzyme involved in hexose dissimilation via this route (see Stewart, 1980; Smith, 1982). There is also evidence that it may be involved in electron transfer to nitrogenase in cyanobacteria providing $NADPH$, from the dissimilation of fixed carbon, which transfers electrons to ferredoxin via ferredoxin- $NADP^+$ oxidoreductase and hence to nitrogenase (Apte *et al.*, 1978). This enzyme occupies a key branch point of carbon metabolism in cyanobacteria and thus its regulation is of particular importance. $NADPH$ (Pelroy *et al.*, 1976; Apte *et al.*, 1978), ATP (Grossman & McGowan, 1975) and light (Duggan & Anderson, 1975) have all been shown to regulate G6PDH and there is evidence that it may be redox-modulated (Czeke *et al.*, 1981). However, the overall regulation of the enzyme is unclear (Schaeffer & Stanier, 1978). Here we provide evidence that thioredoxin may be involved in the modulation of the G6PDH of heterocystous cyanobacteria and consider ways in which this may be of importance.

METHODS

Organism and culture conditions. *Anabaena variabilis* Kütz. ATCC 29413 was grown in axenic culture in one-eighth strength Allen & Arnon's medium (Allen & Arnon, 1955), without combined nitrogen unless stated otherwise, at 25 °C and at a photon flux density incident at the surface of the vessel of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$, in 10 l batch culture or in continuous culture at a dilution rate of 0.03 h^{-1}

Abbreviation: G6PDH, glucose-6-phosphate dehydrogenase.

Preparation of cell-free extracts. Cultures were harvested by centrifugation using a Sharples continuous centrifuge, resuspended and washed twice in 20 mM-Tris/maleate buffer (pH 6.5). Cells were disrupted by passage three times through a French pressure cell at 110 MPa and cell debris was removed by centrifugation at 10000 *g* for 10 min.

Purification of G6PDH. For purification of the enzyme, the cyanobacterium was grown in Allen & Arnon's medium as above, but supplemented with 5 mM-fructose both to increase biomass and provide cells with enhanced G6PDH activity. Cells (about 30 g wet weight) were harvested, washed and resuspended in 20 mM-Tris/maleate buffer (pH 6.5) and disrupted by passage through a French pressure cell as above. The cell-free extract was treated with $(\text{NH}_4)_2\text{SO}_4$ to 35% saturation for 30 min, and then centrifuged at 35000 *g* for 30 min. The resulting supernatant was dialysed against 20 mM-Tris/maleate buffer (pH 6.5) to remove $(\text{NH}_4)_2\text{SO}_4$, then chromatographed on DEAE-cellulose (27 × 2 cm column). The column was eluted with a gradient of 0 to 0.7 M-KCl in 20 mM-Tris/maleate buffer (pH 6.5). Fractions showing highest G6PDH activity were pooled, treated with $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation for 30 min, and then centrifuged at 35000 *g* for 30 min. The resulting precipitate was taken up in a small volume of buffer and dialysed to remove $(\text{NH}_4)_2\text{SO}_4$. Samples (2.5 ml) were chromatographed on Bio-Gel A1.5 m (105 × 1.8 cm column). Active fractions were pooled and concentrated by chromatography on DEAE-cellulose (3 × 1.1 cm column); the enzyme was eluted with 0.15 M-KCl in 20 mM-Tris/maleate buffer (pH 6.5). All operations were carried out at 4 °C.

G6PDH assay (EC 1.1.1.49). Enzyme activity was assayed by following the reduction of NADP⁺ at 340 nm. The reaction mixture contained, in addition to enzyme, in a total volume of 3 ml: 2.2 μmol NADP⁺; 15 μmol glucose 6-phosphate; 30 μmol MgCl₂ and 150 μmol Tris/maleate buffer (pH 6.5).

Protein determinations. The Lowry method was used with bovine serum albumin serving as the standard except for purified enzyme where the turbidimetric method of Mejbaum-Katzenellenboger & Dobryszcka (1959) was used.

Polyacrylamide gel electrophoresis. This was a modification of the method of Davis (1964). Electrophoresis was carried out using vertical slab gels (15 × 15 cm) containing 7.5% (w/v) acrylamide and 0.27% (w/v) *N,N'*-methylenebisacrylamide in 0.1 M-Tris/glycine buffer (pH 8.8), at a current of 30 mA for 2 h. For molecular weight estimations, according to Hedrick & Smith (1968), gels contained 5.5, 6.5, 7.0 or 7.5% (w/v) acrylamide and the molecular weight standards used were pepsin (35000), ovalbumin (46000), bovine serum albumin (68000), yeast glucose-6-phosphate dehydrogenase (128000), and ferritin (440000). Gels were stained for protein with 0.025% (w/v) Coomassie Brilliant Blue R, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid in water for 18 h, followed by intensification in 0.0025% (w/v) Coomassie Brilliant Blue, 10% (v/v) propan-2-ol and 10% (v/v) acetic acid in water. Destaining was in 16.5% (v/v) methanol and 5% (v/v) acetic acid in water.

SDS-polyacrylamide gel electrophoresis. This was done using a 10% (w/v) acrylamide, 0.23% (w/v) *N,N'*-methylenebisacrylamide resolving gel (15 × 15 cm) and a 5% (w/v) acrylamide, 0.13% (w/v) *N,N'*-methylenebisacrylamide stacking gel, according to the method of Laemmli (1970). Standard proteins used were bovine serum albumin (68000), ovalbumin (46000), pepsin (35000), chymotrypsinogen A (25000), trypsin (23300) and lysozyme (14000). Gels were stained and destained as above.

Isoelectric focusing. This was carried out on horizontal slab gels using an LKB multiphor and an LKB 2103 power supply, a suitable pH gradient being formed using LKB ampholine ampholytes. Gels were fixed for 1 h using 11.4% (w/v) trichloroacetic acid, 3.4% (w/v) sulphosalicylic acid and 30% (v/v) methanol in water, stained for protein and destained as above. The pH gradient was determined using a combined surface electrode (Pye Unicam 403–30–M3).

Thioredoxin. Thioredoxin was purified from *Anabaena cylindrica* using a method based on that of Wolosiuk *et al.* (1980). The deactivation of G6PDH and activation of fructose-1,6-bisphosphatase were used to monitor thioredoxin throughout purification. The thioredoxin, which was electrophoretically homogeneous, had a molecular weight of 11800 and an N-terminal amino acid sequence which closely resembled that of other bacterial thioredoxins. Full details of the purification and characterization of this thioredoxin will be published elsewhere.

RESULTS

G6PDH activity of N₂-grown and NO₃⁻-grown Anabaena variabilis

G6PDH in cell-free extracts (10000 *g* supernatants) of *A. variabilis*, which had been grown photoautotrophically in N-free medium in a continuous culture, had a specific activity ($\pm$ SE) of 57.3 ± 3.7 nmol NADP⁺ reduced min⁻¹ (mg protein)⁻¹. When the medium was supplemented with 0.8 mM-NaNO₃, the specific activity was 30.8 ± 4.3 nmol NADP⁺ reduced min⁻¹ (mg protein)⁻¹. The lower activity in extracts of NO₃⁻-grown filaments may have been due to an inhibition of heterocyst production by NO₃⁻ since heterocysts show enhanced G6PDH activity compared with vegetative cells (Winkenbach & Wolk, 1973; Lex & Carr, 1974; Apte *et al.*, 1978). N₂-grown filaments were used in all subsequent experiments.

Fig. 1

Fig. 2

Fig. 1. Effects of DTT and glucose 6-phosphate on the activity of G6PDH of *A. variabilis*. Cell-free extracts of *A. variabilis* (220 μ g protein) were incubated in a volume of 0.2 ml at pH 6.8, without (○) or with (●) 25 mM-DTT. Glucose 6-phosphate was added to a final concentration of 50 mM (□) or 5 mM (■), 5 min after adding DTT. All points are means of at least three replicates with a SE less than $\pm 10\%$.

Fig. 2. Effects of adding DTT or glutamine during the reactivation by glucose 6-phosphate of G6PDH of *A. variabilis* which had previously been deactivated by incubation with NADP^+ . Cell-free extracts of *A. variabilis* (480 μ g protein) were preincubated with 70 μM - NADP^+ at pH 7.5 for 2 min in a volume of 2.95 ml then glucose 6-phosphate was added to 5 mM. Enzyme reactivated in the absence of DTT (○, ●, □); enzyme to which DTT was added to a concentration of 5 mM at the same time as (■) or 2.5 min after (△) the addition of glucose 6-phosphate; and enzyme to which glutamine was added to a concentration of 0.5 mM 2.5 min after the addition of glucose 6-phosphate (▲).

Effects of dithiothreitol, NADP^+ , glucose 6-phosphate and glutamine on G6PDH activity in cell-free extracts

When cell-free extracts of *A. variabilis* were incubated with 25 mM-dithiothreitol (DTT) at pH 6.5 there was a 90% reduction in activity within 5 min (Fig. 1). Deactivation proceeded with apparent first order kinetics, a plot of log activity versus time being linear. Incubation of cell-free extracts with 25 mM-DTT in the presence of 50 μM -glucose 6-phosphate or 0.5 mM-glutamine did not result in a significant decrease in activity compared with a control without glucose 6-phosphate or glutamine. On subsequent addition of glucose 6-phosphate to deactivated enzyme, reactivation occurred rapidly at 50 mM-glucose 6-phosphate but only slowly at 5 mM-glucose 6-phosphate (see Fig. 1); at lower concentration reactivation did not occur. Similar effects of DTT and glucose 6-phosphate on G6PDH of *Plectonema boryanum* have been demonstrated (Raboy *et al.*, 1976).

It has been shown that G6PDH of *Anabaena* 7120 is deactivated on incubation with NADP^+ (Schaeffer & Stanier, 1978). After preincubation of cell-free extracts of *A. variabilis* with 0.7 mM- NADP^+ at pH 7.5 to deactivate the enzyme, activity was restored on adding 5 mM-glucose 6-phosphate (Fig. 2). Deactivation was not observed when the enzyme was preincubated with NADP^+ at pH 6.5. Addition of 5 mM-DTT at various times during reactivation prevented any further increase in activity but did not result in significant deactivation of the enzyme. Addition of 0.5 mM-glutamine during reactivation markedly increased the rate of activation. Thus in cell-free extracts the substrate glucose 6-phosphate and the amino acid glutamine stabilized the enzyme in the activated state whereas DTT or NADP^+ deactivated it, but only in the absence of glucose 6-phosphate or glutamine.

*Involvement of a low molecular weight component in the regulation of *A. variabilis* G6PDH activity*

The deactivation of G6PDH by DTT is in keeping with previous findings for the cyanobacterial enzyme (Duggan & Anderson, 1975). Fig. 3 shows the effect of 25 mM-DTT on G6PDH preparations where the enzyme had been partially purified by gel filtration on

Fig. 3

Fig. 4

Fig. 3. Effects of DTT on G6PDH activity in a partially purified cell-free extract of *A. variabilis* before (○) and after (●) gel filtration of the same preparation on Sephadex G-75 (140 × 1.5 cm column). The cell-free extract was treated with $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation for 30 min and centrifuged at 35000 *g* for 30 min. The supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation for 30 min and then centrifuged at 35000 *g* for 30 min. The resulting pellet was resuspended in a small volume of 50 mM Tris/maleate buffer (pH 6.5) and dialysed against the same buffer for 18 h. Enzyme preparations containing 40 to 70 μg protein with specific activities of 151 and 267 nmol NADP^+ reduced min^{-1} (mg protein) $^{-1}$ (before and after gel filtration respectively) were incubated with 25 mM-DTT at pH 6.5 and 28 °C for the time indicated, before assay.

Fig. 4. Chromatography of a partially purified (see Fig. 3) cell-free extract (4 mg protein) of *A. variabilis* on a 140 × 1.5 cm column of Sephadex G-75. A sample (0.1 ml) of each fraction was assayed for G6PDH activity (○). Samples (0.1 ml) of the fraction showing highest activity (G6PDH was eluted in the void volume) were then assayed, following incubation for 15 min in the presence of 25 mM-DTT plus 0.1 ml of a fraction eluting between 120 ml and 240 ml (●). Molecular weight standards used were: bovine serum albumin, 68000 (1); ovalbumin, 46000 (2); chymotrypsinogen, 25700 (3) and cytochrome c, 12500 (4).

Sephadex G-75 to remove components of lower molecular weight (proteins < 70000 mol. wt). There was no loss of activity over a 60 min period whereas, before gel filtration, activity decreased by 90% within 30 min of adding DTT. The fact that deactivation was slower than that shown in Fig. 1 may be due to the lower concentration of proteins. In any case, a component of the cell-free extract was required to mediate deactivation of G6PDH by DTT. The deactivation of the partially purified enzyme by 25 mM-DTT was restored by preincubating it with samples of the eluate from Sephadex G-75 which would have contained proteins with a molecular weight of approximately 12000 (Fig. 4).

Purification and properties of G6PDH from A. variabilis

In a previous report of the partial purification of G6PDH from the cyanobacterium *Anabaena* 7120 (Schaeffer & Stanier, 1978) sucrose-gradient centrifugation and polyacrylamide gel electrophoresis distinguished three forms with molecular weights of 110000 (M_1), 240000 (M_2) and 345000 (M_3): the hypoactive, normal and hyperactive forms, respectively. G6PDH from *A. variabilis* was purified to electrophoretic homogeneity although in many preparations two minor species of greater electrophoretic mobility were also detected (data not shown); the major species was identified as G6PDH by staining for enzyme activity (Igaue *et al.*, 1981). A single major species ($pI = 4.7$) was observed on isoelectric focusing (Fig. 5). Activity was eluted as a single peak from a column of Bio-Gel A1.5 m. The specific activity was approximately 7.5 μmol NADP^+ reduced min^{-1} (mg protein) $^{-1}$ which represents a 130-fold purification of the enzyme; this compares with a 60-fold purification obtained by Schaeffer & Stanier (1978). Gel filtration on Bio-Gel A1.5 m and electrophoresis in gels of various polyacrylamide concentrations gave a molecular weight of approximately 250000. Polyacrylamide gel electrophoresis in the presence of SDS indicated a single type of polypeptide with a molecular weight of 56200. The purified enzyme thus appears to be a tetramer corresponding to the M_2 form (normal state) observed by Schaeffer & Stanier (1978).

Fig. 5

Fig. 6

Fig. 5. Isoelectric focusing of purified G6PDH (6.4 µg protein). ○, pH.

Fig. 6. Effect of varying the concentration of glucose 6-phosphate on the activity of purified G6PDH.

Fig. 7. Effect of varying the thioredoxin concentration on the rate of deactivation of purified G6PDH of *A. variabilis*. Enzyme (1.6 µg) was incubated together with 2.5 mM-DTT plus purified thioredoxin from *A. cylindrica* at pH 6.5 and 28 °C. The apparent first order rate constants (k_{obs}) were calculated from the plots of log of activity versus time after DTT addition.

The K_m of the enzyme for NADP^+ was 15.5 µM, similar to that obtained previously for G6PDH from various sources (Levy, 1979). The plot of activity against NADP^+ concentration was hyperbolic, showing typical Michaelis–Menten kinetics. The graph of the effect of glucose 6-phosphate concentration on G6PDH, on the other hand, was sigmoidal (Fig. 6), indicative of positive cooperativity in the binding of glucose 6-phosphate or of the presence of both active and inactive forms of the enzyme. At the pH used (6.5) G6PDH would be expected to be in the normal (active) state (Schaeffer & Stanier, 1978).

Effect of thioredoxin on the activity of purified G6PDH

We have demonstrated the presence in *A. variabilis* of a low molecular weight protein(s) (approximately 12000 mol. wt) which deactivated G6PDH. There is also evidence that G6PDH of higher plant chloroplasts is modulated by the protein thioredoxin (Ashton *et al.*, 1980; Scheibe & Anderson, 1981). It seemed possible, therefore, that the factor involved in the regulation of G6PDH was thioredoxin or glutaredoxin, both of which have molecular weights of about 12000 (Holmgren, 1976; Tsang, 1981). Our finding that reduced glutathione at concentrations up to 20 mM (data not shown), unlike DTT, did not deactivate G6PDH suggested that the factor was likely to be thioredoxin rather than glutaredoxin. We therefore examined the effect of various concentrations of purified cyanobacterial thioredoxin on the rate of deactivation of purified G6PDH by DTT in the absence of substrates (Fig. 7). DTT alone did

not cause deactivation, as noted above for the partially purified enzyme. However, when thioredoxin concentrations as low as 2 nM were used together with DTT (2.5 mM), deactivation of G6PDH occurred with apparent first order kinetics. At thioredoxin concentrations greater than 32 nM, the rate at which the reaction proceeded to completion was too rapid to allow its accurate measurement. Reduced glutathione (data not shown) had no effect on G6PDH activity at concentrations up to 20 mM in the presence of thioredoxin. Thus, deactivation of G6PDH from the N_2 -fixing cyanobacterium *A. variabilis* by a thioredoxin-mediated process clearly occurs.

DISCUSSION

Glucose-6-phosphate dehydrogenase (G6PDH), both in higher plants (Wildner, 1975; Igaue *et al.*, 1981) and cyanobacteria (Schaeffer & Stanier, 1978), is a hysteretic enzyme which can exist in an active associated form or in an inactive dissociated form. There is good evidence from work on cyanobacteria that, in vegetative cells, G6PDH activity is light-modulated, with activity being lower in the light than in the dark. Such an inhibition/deactivation in the light has been explained in terms of allosteric regulation by ribulose 1,5-bisphosphate (Pelroy *et al.*, 1972; Schaeffer & Stanier, 1978), by ATP (Grossman & McGowan, 1975) and by NADPH (Pelroy *et al.*, 1976; Apte *et al.*, 1978), all of which are generated photosynthetically. The equilibrium between the active and inactive forms of the enzyme can be affected by metabolites such as $NADP^+$, glucose 6-phosphate and glutamine and by physicochemical factors such as pH (Schaeffer & Stanier, 1978).

We have shown in this paper that the *A. variabilis* enzyme, like that of *Anabaena* 7120 (ATCC 27893; Schaeffer & Stanier, 1978) and *A. cylindrica* (Apte *et al.*, 1978) may be regulated *in vitro* by $NADP^+$ and glucose 6-phosphate. Also important, however, is our finding that in *A. variabilis* G6PDH is regulated by thioredoxin. Thioredoxins are small proteins (mol. wt ~ 12000) which have been implicated in enzyme activation/deactivation in plants and cyanobacteria (Buchanan *et al.*, 1979); other regulatory proteins are the light effect mediator system (Mohammed & Anderson, 1981) and ferraltherin (Lara *et al.*, 1980; de la Torre *et al.*, 1982). Most attention has been paid to their effects on fructose-1,6-bisphosphatase and other enzymes of the Calvin-Benson cycle. The effect of thioredoxin on G6PDH of higher plants has been reported by Scheibe & Anderson (1981) and there is a recent report of thioredoxin-mediated deactivation of G6PDH in the unicellular cyanobacterium *Anacystis nidulans* (Udvardy *et al.*, 1983).

The thioredoxin used in this investigation was from *Anabaena cylindrica*. This purified thioredoxin, at concentrations as low as 2 nM, deactivated purified *A. variabilis* G6PDH indicating a potential regulatory role for this protein in heterocystous N_2 -fixing cyanobacteria. The deactivation required reduced thioredoxin, which was produced *in vitro* by adding DTT but, *in vivo*, reduction probably depends on reduction of thioredoxin by ferredoxin-thioredoxin oxidoreductase (Yee *et al.*, 1981). The precise mechanism of deactivation may involve reduction of an enzyme disulphide, or thioredoxin may catalyse an intramolecular thiol-disulphide exchange involving a cysteine and a disulphide in the enzyme (Anderson *et al.*, 1978).

The involvement of thioredoxin in G6PDH modulation has important implications in N_2 -fixing cyanobacteria and adds a new dimension to our understanding of the regulation of this key enzyme of carbon metabolism in cyanobacteria in general. In vegetative cells thioredoxin may play a key role in the light deactivation/dark activation of the oxidative pentose phosphate pathway (see Winkenbach & Wolk, 1973; Lex & Carr, 1974; Apte *et al.*, 1978). In the light photosynthetically produced reductant may serve to sustain a supply of reduced thioredoxin resulting in deactivation of G6PDH and thus of the oxidative pentose phosphate pathway. In the dark, when light-generated reductant would not be available and thioredoxin would not be so reduced, G6PDH would be activated and the oxidative pentose phosphate pathway would function. Activation could also involve glucose 6-phosphate (see Figs 1 and 2) derived from fixed carbon reserves such as glycogen.

In heterocysts, unlike vegetative cells, G6PDH is active in both the light and dark (Winkenbach & Wolk, 1973; Lex & Carr, 1974; Apte *et al.*, 1978). Our data suggest two possible reasons for this, neither of which is mutually exclusive. First, there may be insufficient reduced

thioredoxin available in heterocysts to inactivate G6PDH in the light, either because of a lack in heterocysts of a functional thioredoxin/thioredoxin reductase system or because in the light, when photosynthetically generated ATP becomes available both for nitrogenase and glutamine synthetase, there is a diversion of reductant to these enzymes of nitrogen metabolism at the expense of thioredoxin. Schmidt & Christen (1979) have reported higher thioredoxin levels in a heterocystous cyanobacterium grown on N_2 when compared with NO_3^- -grown filaments which may indicate that thioredoxin is present in heterocysts. Second, we have shown that the effects of glucose 6-phosphate, glutamine, $NADP^+$ and reduced thioredoxin on G6PDH cannot be viewed in isolation, with $NADP^+$ and reduced thioredoxin promoting deactivation of the enzyme whereas glucose 6-phosphate and glutamine prevented deactivation and, under certain conditions, promoted reactivation. It is possible that, in heterocysts, glutamine, which is probably the form in which fixed nitrogen is transported from heterocysts (Thomas *et al.*, 1977), accumulates in quantity and, together with glucose 6-phosphate, provided directly or indirectly from vegetative cells, serves to override the deactivating effect of reduced thioredoxin.

This work was supported by the Science and Engineering Research Council. We thank S. M. Ip for providing purified thioredoxin.

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