Multiple oligomeric forms of glucose-6-phosphate dehydrogenase in cyanobacteria and the role of OpcA in the assembly process

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Multiple molecular forms of glucose-6-phosphate dehydrogenase (G6PDH) were detected by activity staining in non-denaturing polyacrylamide gels of cell-free extracts from a range of cyanobacteria including *Anabaena* sp. PCC 7120, *Synechococcus* sp. PCC 7942, *Plectonema boryanum* PCC 73110, *Synechocystis* sp. PCC 6803, *Nostoc* sp. MAC PCC 8009 and the marine strain *Synechococcus* sp. WH7803. In most of the species tested, the profile of G6PDH activities was modulated by the growth of the cells in the presence of exogenous 10 mM glucose. Using an antiserum raised against a fragment of G6PDH from *Anabaena* sp. PCC 7120, it was shown that the different molecular forms of G6PDH all contained an antigenically related subunit, suggesting that the different forms arose from different quaternary structures involving the same monomer. An insertion mutant of *Synechococcus* sp. PCC 7942 was constructed in which the *opcA* gene, adjacent to *zwf* (encoding G6PDH), was disrupted. Although no reduction in the amount of G6PDH monomers (*Zwf*) was observed in the *opcA* mutant, activity staining of native gels indicated that most of this protein is not assembled into one of the active oligomeric forms. The oligomerization of G6PDH in extracts of the *opcA* mutant was stimulated *in vitro* by a factor present in crude extracts of the wild-type, suggesting that the product of the *opcA* gene is involved in the oligomerization and activation of G6PDH.

Keywords: cyanobacteria, glucose-6-phosphate dehydrogenase, oligomeric protein, chaperone

INTRODUCTION

Cyanobacteria are a physiologically cohesive group of organisms that exhibit oxygenic photosynthesis and for which photoautotrophy is the dominant nutritional mode. In the natural environment, cyanobacteria are subject to a diurnal light–dark cycle, during which the organisms must switch between the photoautotrophic metabolic mode, involving the reductive pentose phosphate pathway (RPP) for growth and a dark heterotrophic mode for cell maintenance. Although enzymes of both the glycolytic and oxidative pentose phosphate

(OPP) pathways have been detected in representative species, relative specific activities as well as respiratory studies using glucose radioactively labelled in the C1 and C6 positions have favoured the OPP as the most important route of dark oxidative glucose dissimilation (see Carr, 1973; Smith, 1982). The OPP is thought also to be largely responsible for the supply of reductant to nitrogenase in the heterocyst (Apte *et al.*, 1978; Summers *et al.*, 1995b). However, the importance of the OPP in carbohydrate metabolism in cyanobacteria becomes less clear when the properties of insertional mutants of the gene (*zwf*) encoding the key enzyme of the OPP, glucose-6-phosphate dehydrogenase (G6PDH), are considered. Wild-type and *zwf* insertional mutants of *Synechococcus* sp. PCC 7942 (*Anacystis nidulans*) exhibited a similar growth rate and dark respiration rate; indeed, the only detectable phenotype of the *zwf* strain was a loss of viability during prolonged incubation in the dark

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; OPP, oxidative pentose phosphate pathway; RPP, reductive pentose phosphate pathway.

(Scanlan *et al.*, 1995). Earlier, Doolittle & Singer (1974) had observed that a double mutant of *A. nidulans* lacking 6-phosphogluconate dehydrogenase and G6PDH also exhibited significant dark oxygen uptake. Summers *et al.* (1995b) have demonstrated that a *zwf* mutant of *Nostoc* sp. ATCC 29133, a photoheterotrophic cyanobacterium, could no longer grow in the dark at the expense of fructose or grow under any conditions as a diazotroph. Thus, G6PDH has a central, but under some conditions replaceable, role in the carbohydrate metabolism of these organisms. The mechanism involved in the switch from the RPP to the OPP remains obscure, but much interest has been focused on the regulation of G6PDH. Metabolites including NADPH (Pelroy *et al.*, 1976; Apte *et al.*, 1978) and ATP (Grossman & McGowan, 1975) have been implicated in regulation, and thioredoxin control via a thiol/disulphide exchange has also been proposed by both Cossar *et al.* (1984) and Udvardy *et al.* (1984). This latter proposed mechanism has received support from the observation that two cysteine residues are absolutely conserved in all the cyanobacterial *zwf* genes so far sequenced (Newman *et al.*, 1995). The sensitivity of G6PDH to thioredoxin control may be markedly affected by pH (Gleason, 1996). Another process may also be implicated in determining G6PDH activity. Immediately downstream from the *zwf* gene in *Nostoc* sp. ATCC 29133 (Summers *et al.*, 1995a) and *Synechococcus* sp. PCC 7942 (Newman *et al.*, 1995) is a gene designated *opcA*. The *zwf* and *opcA* genes are unlinked in *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996). It has been shown for *Nostoc* sp. ATCC 29133 that *opcA* is co-transcribed with *zwf* (Summers & Meeks, 1997) and that mutations in *opcA* cause a phenotype very similar to that observed for a *zwf* mutant, with an almost complete loss of G6PDH activity and loss of the ability to grow on exogenous fructose or diazotrophically (Summers *et al.*, 1995b). Finally, Schaeffer & Stanier (1978) have proposed that cyanobacterial G6PDHs may exist in a range of catalytic/aggregation states and that the transitions between such states may determine enzyme activity. The occurrence of different G6PDH aggregation states with differing enzymic properties has been confirmed more recently by Gleason (1996). In this paper we document the existence of different oligomeric forms of G6PDH in crude extracts of cyanobacteria and examine the possible role of the *opcA* gene in the oligomer assembly process.

METHODS

Organisms and growth conditions. The freshwater cyanobacterial strains *Anabaena* sp. PCC 7120, *Synechococcus* sp. PCC 7942, *Plectonema boryanum* PCC 73110, *Synechocystis* sp. PCC 6803 and *Nostoc* sp. MAC PCC 8009 were grown in BG11 and BG11₀ media (Rippka *et al.*, 1979) as appropriate, under continuous illumination (20 $\mu\text{E m}^{-2} \text{s}^{-1}$) from warm white fluorescent tubes (Osram) at 30 °C. The marine strain *Synechococcus* sp. WH7803 was grown in artificial-sea-water medium (Wyman *et al.*, 1985) at 25 °C under similar illumination conditions. Where stated, glucose and the non-metabolizable analogues 2-deoxy-D-glucose and 3-O-methyl-

D-glucose were added to the medium to a final concentration of 10 mM, and chloramphenicol was added to 100 $\mu\text{g ml}^{-1}$. *Escherichia coli* strains were grown in LB medium and 2 $\times$ YT medium (Sambrook *et al.*, 1989) as appropriate. Ampicillin and kanamycin were used at 50 and 25 $\mu\text{g ml}^{-1}$, respectively.

Preparation of cell-free extracts and assay of G6PDH activity.

Cyanobacterial cultures (1 l) were harvested by centrifugation (10 min, 10000 g, 4 °C), the cells were washed once and resuspended in 1 ml 50 mM Tris/maleate buffer, pH 6.5, 10 mM glucose 6-phosphate, 0.1% β -mercaptoethanol (Schaeffer & Stanier, 1978). Where appropriate, β -mercaptoethanol was omitted from the buffer and/or glutamine (0.1, 1.0, 5.0 or 10 mM) was included. Cells were disrupted using an Aminco French pressure cell at a closing pressure of 20000 p.s.i. (138 MPa). Four passes were routinely required to give >90% cell breakage. Cell-free extracts were then prepared by high-speed centrifugation (80000 g, 30 min, 4 °C) and the supernatants were kept at 4 °C and used on the day of preparation. G6PDH (EC 1.1.1.49) was assayed in cell-free extracts as described by Scanlan *et al.* (1995). Protein was assayed by the Lowry method.

General molecular biology techniques. Plasmid isolation from *E. coli*, restriction digestion, ligation using T4 ligase and transformations into *E. coli* were performed using standard molecular biological techniques (Sambrook *et al.*, 1989). Ligation reactions were performed in 20 μl volumes with 500 ng DNA and 0.5 U T4 ligase (Gibco-BRL) at 15 °C for 18 h. *Synechococcus* sp. PCC 7942 cells were transformed as described previously (Scanlan *et al.*, 1990).

Insertional mutagenesis of *opcA*. Plasmid pDB is a derivative of pUC19 and contains a *SalI* fragment of approximately 6 kb from *Synechococcus* sp. PCC 7942 DNA, which carries the 3' end of the *zwf* gene, the complete *opcA* gene, and two further genes encoding cytochrome *b₆* and subunit IV of the cytochrome *b₆f* complex (Newman *et al.*, 1995). There are two *PstI* sites in pDB: one in the multiple-cloning site and one 300 bp downstream of the *opcA* gene. Digestion of pDB with *PstI* yielded two fragments, one of which (4.8 kb) contained 2.2 kb of the original insert, including the intact *opcA* gene, together with pUC19. This fragment was self-ligated to produce pSDG2. This subclone has a unique *BglII* site approximately in the middle of the *opcA* gene. Plasmid pHP45 Ω carries the 2 kb Ω fragment consisting of the antibiotic resistance gene *aadA*, conferring resistance to streptomycin and spectinomycin, flanked by short inverted repeats carrying transcription and translation termination signals and synthetic polylinkers including *BamHI* sites (Prentki & Krisch, 1984). The Ω fragment was produced by *BamHI* digestion of pHP45 Ω and was inserted into the compatible *BglII* site of pSDG2, thereby interrupting the *opcA* gene and yielding plasmid pSDG4 (*opcA*:: Ω).

Synechococcus sp. PCC 7942 cells were transformed with pSDG4 and grown under selection for streptomycin resistance. Of 50 Sm^r transformants screened, two were found to be ap^r and were, therefore, presumed to have arisen by integration of pSDG4 into the chromosome by a single crossover. Those clones expressing only streptomycin resistance were assumed to have arisen via a double crossover in which the chromosomal copy of the *opcA* gene was replaced by the mutated copy from pSDG4. A single recombinant clone (HK52) and one double recombinant clone (HK55) were selected for further analysis by Southern blotting to confirm their structure.

Heterologous gene expression. PCR amplification of a portion of the *Anabaena* sp. PCC 7120 *zwf* gene was carried out using a forward primer (5'-CCCGGATCCTAGAAAA-

TCCCTTGCG-3') and a reverse primer (5'-CCCGGATCC-ACGCCTACGGTTTCTGC-3'). These primers correspond to amino acid residues 4–10 (forward) and 240–234 (reverse) (Newman *et al.*, 1995) and were designed to yield *Bam*HI sites at each end of the amplified product. Amplification of the 720 bp fragment was carried out in a reaction volume of 50 μ l using *Taq* polymerase (Promega) and buffers supplied by the manufacturer, with 50 ng template DNA and 100 pmol of each primer. The Mg^{2+} concentration was 2.5 mM and the reactions were carried out in a Hybaid Omnigene Thermocycler with 25 cycles of 94 °C for 1 min, 40 °C for 2 min and 72 °C for 2 min. Reaction products were checked for size and purified from 0.7% agarose gels using the GeneClean kit (Bio101). The purified products were digested with *Bam*HI and inserted into the *Bam*HI site of expression vector pQE32 (Qiagen). After transformation into *E. coli*, several transformants were picked and the relevant portion of the plasmids sequenced to confirm the integrity of the inserted PCR fragment. DNA sequencing reactions were carried out using the Sequenase 2.0 Sequencing kit (USB) according to the manufacturer's instructions. One clone was selected for purification of the expressed 31 kDa G6PDH fragment, following induction by IPTG. The pQE32 vector provides an N-terminal fusion of six histidine residues to the expression product to facilitate one-step purification of the polypeptide by metal-chelate chromatography. The 31 kDa *Anabaena* sp. PCC 7120 G6PDH fragment overexpressed in *E. coli* was purified using Ni-NTA (Qiagen) chromatography as recommended by the manufacturer.

Antibody production. Approximately 75 μ g of the purified *Anabaena* sp. PCC 7120 G6PDH polypeptide fragment overexpressed in *E. coli* was used to raise an anti-G6PDH antiserum in a Sandy Half Lop rabbit. The immunization schedule was as previously described by Scanlan *et al.* (1989).

SDS-PAGE, Western blotting, non-denaturing PAGE and enzyme activity staining. SDS-PAGE in 10% slab gels was carried out as described by Hames (1981). Western blotting was carried out as described by Silman *et al.* (1995) and blots were developed, following treatment with the primary antibody (diluted 1:1000), by conventional methods, using a horseradish peroxidase-linked secondary antibody (Amersham). G6PDH was detected in non-denaturing PAGE gels as described by Scanlan *et al.* (1995). Non-denaturing polyacrylamide gradient gels (4–30%) for G6PDH activity staining were made as described previously (Scanlan *et al.*, 1995) and the samples (200 μ g protein, except where stated otherwise) of fresh cell-free extract were loaded onto the gel without prior heating.

Cell viability analysis. Dark viability of wild-type and *opcA* mutant cells was assayed by dilution plating onto BG11 plates. Flasks containing light-grown mutant and wild-type cells were wrapped with foil to exclude light and sampled daily, with dilutions being plated onto BG11 agar and incubated at 30 °C under constant illumination. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Detection of multiple G6PDH activities

Schaeffer & Stanier (1978) showed that, *in vitro*, extensively purified G6PDH from *Anabaena* sp. PCC 7120 (= *Anabaena* sp. ATCC 27893) underwent relatively slow, reversible transitions between different aggregation states with apparent molecular masses of 120 (M1), 240 (M2) and 345 (M3) kDa, which differed in

Fig. 1. Effect of exogenous 10 mM glucose on the appearance of novel molecular forms of G6PDH in a range of cyanobacteria. Cell-free extracts (200 μ g per lane) from cultures grown in either the absence or presence of exogenous 10 mM glucose were subjected to electrophoresis in a gradient (4–30%) non-denaturing polyacrylamide gel, which was subsequently stained for G6PDH activity. The second of the two lanes in each pair contained glucose in the growth medium. The specific activities of the extracts are indicated in parentheses after the strain name; the units (U) of specific activity are nmoles NADP reduced $mg^{-1} min^{-1}$. Lanes 1 and 2, *Anabaena* sp. strain PCC 7120 (– glucose, 282 U; + glucose, 300 U); lanes 3 and 4, *Plectonoma boryanum* strain PCC 73110 (165 U; 205 U); lanes 5 and 6, *Synechocystis* sp. strain PCC 6803 (220 U; 283 U); lanes 7 and 8, *Nostoc* sp. MAC strain PCC 8009 (192 U; 208 U); lanes 9 and 10, *Synechococcus* sp. strain PCC 7942 (232 U; 256 U); lanes 11 and 12, *Synechococcus* sp. strain WH7803 (275 U; 246 U). The positions and sizes of the protein standards are indicated on the left of the gel, and the relative positions of the *Anabaena* sp. strain PCC 7120 activity bands (M1, M2, M3) are shown on the right.

their catalytic activity ($M1 \ll M2 < M3$). More recently, Gleason (1996) has shown that the aggregation states are markedly pH dependent. However, the work was conducted with enzyme preparations which had undergone several lengthy purification steps and were comparatively dilute; consequently, inferences regarding the physiological significance of these observations could only be tentative. The first objective of this study was to establish whether multiple molecular forms of G6PDH could be detected in unpurified, crude cell extracts and whether the profile of molecular forms was modulated by environmental factors. This would provide more direct evidence for the physiological significance of such multiple aggregation/catalytic states. The approach adopted was to carry out activity staining of G6PDH following native PAGE of crude cell-free extracts.

Initial experiments with *Anabaena* sp. PCC 7120 indicated that several distinct molecular forms of G6PDH activity could be detected by this approach. The analysis was extended to a range of cyanobacteria including other filamentous, heterocystous strains, and unicellular freshwater and marine strains. The effect of one environmental factor, namely the presence of exogenous

10 mM glucose in the medium, was also investigated. It is clear from Fig. 1 that in the case of *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803, *Nostoc* sp. MAC PCC 8009, *Synechococcus* sp. PCC 7942, there are multiple bands of G6PDH activity exhibiting different mobilities on a native PAGE gel, and also that one or more novel forms of the enzyme appear in response to exogenous glucose. In the case of *Anabaena* sp. PCC 7120, the non-metabolizable analogues 2-deoxy-D-glucose and 3-O-methyl-D-glucose did not exert the same effect (data not shown). The calculation, on the basis of mobility, of the apparent size of protein bands in native gels is unreliable, because the rate of migration is determined not only by the size, but also by the overall charge on the protein at the pH of the gel buffer (pH 8.8) and the buffering capacity in the protein sample. Consequently, there was some variability in the estimations of the size of the three activity bands detected in extracts of *Anabaena* sp. PCC 7120, particularly with respect to the largest size band. The mean sizes of the activity bands were 380 (range 320–500), 200 (range 185–210) and 107 (range 96–140) kDa. The sizes of these bands and the values reported by Schaeffer & Stanier (1978) (M3, 345 kDa; M2, 240 kDa; M1, 120 kDa) are in reasonable agreement and are consistent with hexameric, tetrameric and dimeric forms of an approximately 58 kDa monomer subunit; the three forms are consequently referred to as M3, M2 and M1. The induction of additional molecular forms of G6PDH, similar to those observed in response to exogenous glucose, was observed for *Anabaena* sp. PCC 7120 following a transition to diazotrophic growth (data not shown). *Plectonema boryanum* PCC 73110 exhibited several rather weak activity bands, but there was little observable difference when the cells were grown with glucose in the medium. In the case of the marine phycoerythrin-containing *Synechococcus* sp. WH7803 there were again multiple forms, but the presence of glucose caused the disappearance of one species of approximately 250 kDa. The induction by exogenous glucose of the novel molecular forms of G6PDH in *Anabaena* sp. PCC 7120 had a time course of approximately 18 h and could be blocked by chloramphenicol (data not shown).

In the context of the response of the organisms used here to exogenous glucose, the following nutritional characteristics should be noted. *Nostoc* sp. MAC PCC 8009 (Ingram *et al.*, 1973) and *Synechocystis* sp. PCC 6803 (Astier *et al.*, 1984; Anderson & McIntosh, 1991) are capable of chemoheterotrophic growth; the other freshwater strains are obligate photoautotrophs or facultative photoheterotrophs (Rippka *et al.*, 1979). Thus, there is no correlation between heterotrophic capacity and the possession or induction of multiple molecular forms of G6PDH. It should be noted that glucose can make a major (46%) contribution to the dry weight of cellular material, even in obligately photoautotrophic organisms such as *Anabaena variabilis* (*Anabaena* sp. PCC 7118) (Pearce & Carr, 1969).

Given the reported redox sensitivity of G6PDH in

Fig. 2. Western blot of an SDS-polyacrylamide gel of total SDS-soluble protein from wild-type *Synechococcus* sp. PCC 7942 cells and *opcA* mutants. The cells were grown in the absence of glucose in all cases. The blot was probed with an antibody raised against a fragment of the *Anabaena* sp. PCC 7120 G6PDH and visualized with a horseradish-peroxidase-linked secondary antibody. Lanes: 1, wild-type; 2, single recombinant mutant strain; 3, double recombinant mutant strain. The positions and sizes of the protein standards are indicated on the left and the calculated size (kDa) of the reacting band on the right.

cyanobacteria (Cossar *et al.*, 1984; Udvardy *et al.*, 1984) and the stabilizing effect of glutamine (Schaeffer & Stanier, 1978; Rowell & Simpson, 1990) extracts of *Anabaena* sp. PCC 7120 were made using Tris/maleate buffer lacking β -mercaptoethanol and/or containing glutamine (0.1, 1.0, 5.0 or 10.0 mM). However, these modifications to the extraction buffer had no effect on the G6PDH activity profiles from cultures grown in the presence or absence of exogenous glucose (data not shown).

Presence of the Zwf subunit in the different molecular forms of G6PDH

To confirm the hypothesis that all forms of the enzyme were composed of the same subunits, an antibody was raised against the *zwf* monomer encoded by the gene from *Anabaena* sp. strain PCC 7120 (Newman *et al.*, 1995). The antiserum specifically recognized a single polypeptide of approximately 53 kDa in a Western blot of an SDS-polyacrylamide gel of a cell extract from *Anabaena* sp. strain PCC 7120, and exhibited no detectable cross-reaction with any other polypeptides (Fig. 2). This antiserum was used to probe a Western blot of a native gel of proteins from extracts of *Anabaena* sp. strain PCC 7120 cells, grown in the presence or absence of 10 mM glucose (Fig. 3). Comparison with the activity-stained duplicate tracks from the same gel confirmed that the novel forms of G6PDH which appeared in response to exogenous glucose did, indeed, contain monomers which were immunologically cross-reactive. Given the specificity of the antibody, it can be concluded that the multiple forms contained the same catalytic subunit and differed only with respect to the aggregation state. However, the possibility cannot be

Fig. 3. Western blot analysis of the novel molecular forms of G6PDH induced in *Anabaena* sp. strain PCC 7120 in response to exogenous glucose. Cell-free extracts from cultures grown in either the presence or absence of exogenous 10 mM glucose were analysed on a gradient (4–30%) non-denaturing polyacrylamide gel. One half of the gel was stained for G6PDH activity and the other half was used for Western blot analysis using the antibody raised against the truncated G6PDH-6×His fusion polypeptide. The second lane from each pair contained glucose in the growth medium. Lanes: 1 and 2, G6PDH activity stained; 3 and 4, Western blot. The positions and sizes of the protein standards are indicated on the left of the gel and the positions of the M1, M2 and M3 activity bands are indicated on the right.

excluded that there is an additional polypeptide associated with the catalytic subunits that is involved in determining the aggregation state. Experiments where the activity bands were cut out of native gels and re-analysed on SDS-polyacrylamide gels did not give a clear answer to this question, as the amount of protein in some of the alternative forms was very low and other proteins overlapped with G6PDH in the native gels (data not shown).

Insertional mutagenesis of *opcA* in *Synechococcus* sp. PCC 7942

Immediately downstream from the *zwf* gene in *Nostoc* sp. strain ATCC 29133 (Summers *et al.*, 1995a) and *Synechococcus* sp. PCC 7942 (Newman *et al.*, 1995) is a gene designated *opcA* encoding a protein of unknown function. In *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) the *zwf* and *opcA* genes are unlinked. It has been

shown for *Nostoc* sp. strain ATCC 29133 that *opcA* is co-transcribed with *zwf* and that mutations in *opcA* cause an almost complete loss of G6PDH activity (Summers *et al.*, 1995b). We were interested in establishing whether the *opcA* gene product played any part in determining the oligomeric state of G6PDH. Insertional mutagenesis of the *opcA* gene was performed in *Synechococcus* sp. PCC 7942 since this strain is easily genetically manipulable and the *opcA* gene had already been cloned from it (Newman *et al.*, 1995). Transformation of *Synechococcus* sp. PCC 7942 with pSDG4, in which the *opcA* gene was inactivated by insertion of the Ω fragment (see Methods), led to the isolation of a single recombinant clone (HK52), in which the plasmid had become integrated into the chromosome, and a double recombinant clone (HK55), in which the chromosomal copy of *opcA* had been replaced by the mutated copy. In the case of HK52, the single recombination event appeared to have occurred upstream of the Ω fragment; thus the mutated copy of the *opcA* gene would lie between *zwf* and the unmutated copy of *opcA*. Therefore, there may be some residual transcription of the unmutated *opcA* gene in the single recombinant if it has its own functional promoter.

Assay of G6PDH in cell-free extracts revealed that the double recombinant (HK55) exhibited an activity of 1.6 nmol NADPH formed $\text{min}^{-1} \text{mg}^{-1}$, which represents 1.4% of wild-type activity (110.9 nmol NADPH formed $\text{min}^{-1} \text{mg}^{-1}$), and the single recombinant exhibited 24% of wild-type activity. The growth rates of the two mutants were similar to that of the wild-type under photoautotrophic growth conditions (data not shown). After 72 h incubation in the dark, viability of the double recombinant (0%) was markedly reduced compared to the wild-type (47%); however, viability of the single recombinant was similar to that of the wild-type. Thus, the phenotypic effects of mutagenesis of *opcA* were similar to those previously observed for *zwf* mutants of *Synechococcus* sp. PCC 7942 (Scanlan *et al.*, 1995) and in agreement with the results previously obtained with an *opcA* mutant of *Nostoc* sp. strain ATCC 29133 (Summers *et al.*, 1995b). Western blotting was employed to establish whether expression of the *zwf* gene was affected by the mutation in *opcA*. The antibody produced against an internal fragment of G6PDH from *Anabaena* sp. PCC 7120 was used to probe Western blots of an SDS-polyacrylamide gel of cell extracts from the wild-type and strains HK52 (single recombinant) and HK55 (double recombinant) (Fig. 2). No significant reduction of the amount of the G6PDH polypeptide was detected in either the single or double recombinant, indeed a slight increase is apparent in the mutants, suggesting that transcription, translation and stability of *zwf* was not significantly reduced.

Effect of the *opcA* mutation on the quaternary structure of G6PDH

The effects of the *opcA* mutations on the oligomeric structure of G6PDH were examined by activity staining following non-denaturing PAGE analysis of cell-free

Fig. 4. A G6PDH-activity-stained non-denaturing 10–30% polyacrylamide gel comparing activities in cell-free extracts (200 µg per track) from wild-type cells and from *opcA* insertion mutants. The (+) or (–) after the lane number indicates the presence or absence of glucose in the growth medium. Lanes 1 (–) and 2 (+), *Anabaena* sp. PCC 7120; lanes 3 (–) and 4 (+), *Synechococcus* sp. PCC 7942 wild-type; lane 5 (–), *Synechococcus* sp. PCC 7942 *opcA* single recombinant; lane 6 (–), *Synechococcus* sp. PCC 7942 *opcA* double recombinant. The positions and sizes of the protein standards are indicated on the left of the gel and the positions of the *Anabaena* sp. PCC 7120 M1, M2 and M3 activity bands are indicated on the right.

extracts (Fig. 4). This approach revealed that the activity bands observed in *Synechococcus* sp. PCC 7942 were broadly similar in size to those (M1, M2, M3) obtained with *Anabaena* sp. PCC 7120, and consequently this terminology is retained. There was a band of activity (M3) in wild-type *Synechococcus* sp. PCC 7942 extract, with a size greater than 440 kDa (Fig. 4). Exogenous glucose induced two new activities (M2 and M1) with apparent sizes of 210 kDa and 140 kDa. The single recombinant also showed M3, but with reduced activity compared to the wild-type. However, a novel form of G6PDH activity was apparent at approximately 120 kDa, corresponding to the M1 activity band induced by exogenous glucose. The double recombinant gave a drastically reduced activity of M3 as well as M1. Thus, although the monomers of G6PDH were being synthesized in approximately normal amounts in the *opcA* mutant, assembly into the active oligomeric forms was greatly reduced.

Assembly of G6PDH in cell-free extracts

There are a variety of ways in which OpcA may be involved in determining the oligomeric state of G6PDH; the most direct would be an interaction (possibly stoichiometric) between OpcA and the G6PDH monomer. To establish evidence for such a protein–protein interaction, the following approach was adopted. Cell-free extracts of wild-type *Synechococcus* sp. PCC 7942 will contain OpcA, whereas cell-free extracts from the *opcA* mutant (double recombinant) should contain G6PDH monomers, but no OpcA. A mixture of the two

Fig. 5. Activation of G6PDH in an extract from cells of a *Synechococcus* sp. PCC 7942 *opcA* mutant by the addition of wild-type cell-free extract in a 1:4 ratio (on a protein basis). The individual and mixed extracts were incubated at 30 °C for 60 min prior to loading onto a non-denaturing 10–30% PAGE gel followed by electrophoresis and detection of G6PDH by activity staining. Lanes: 1, wild-type extract (40 µg); 2, *opcA* mutant extract (160 µg); 3, mixed extracts (200 µg). The positions and sizes of the protein standards are indicated on the left of the gel.

cell extracts might permit assembly of monomers into active multimers with an increase in the total activity over the sum of that of the two individual extracts. Attempted activation of G6PDH activity was carried out by mixing wild-type and *opcA* mutant extracts in a 1:4 ratio (on a protein basis) and incubating for 60 min at 30 °C prior to native gel electrophoresis and activity staining (Fig. 5). It is clear that there is much greater G6PDH activity in the high-molecular-mass form in the lane containing the mixed extracts than in either individual extract. Thus, the unassembled G6PDH subunits in the *opcA* mutant extract could apparently be assembled into a catalytically active oligomeric form, presumably via an interaction with OpcA in the wild-type extract. Heterologous activation of G6PDH subunits in the cell-free extract of the *opcA* mutant was also observed following incubation with cell-free extracts from either *Anabaena* spp. Thus, it would appear that OpcA is directly involved in determining the oligomeric state of G6PDH, though the stoichiometry of the process and nature of the interaction remain to be established.

Conclusions

It is apparent from these results that G6PDH from cyanobacteria can exist in multiple molecular forms, presumably representing different aggregation states of

the same catalytic monomer. The fact that changes in the *in vitro* profile of G6PDH molecular forms could be modulated by exposure of the cells to environmental factors, such as exogenous glucose, argues strongly that the different molecular forms are of significance *in vivo*. At this stage, the physiological role(s) of these molecular forms is unclear; however, it is tempting to speculate that it may be related to the various regulatory properties of the enzyme in relation to pools of various metabolites (Grossman & McGowan, 1975; Pelroy *et al.*, 1976; Apte *et al.*, 1978) as well as the proposed thiol/disulphide exchange (Cossar *et al.*, 1984; Udvardy *et al.*, 1984) or pH (Gleason, 1996).

The product of the *opcA* gene is involved, directly or indirectly, in establishing the oligomerization of the G6PDH monomer into its catalytically active multimeric state. This result explains the previous observation that mutations within the *opcA* gene cause loss of almost all G6PDH activity. The residual G6PDH activity shown in *opcA* mutants may result from a limited ability of the G6PDH monomer to self assemble, or from the presence of a protein of OpcA-related function, which can, to a limited extent, complement the lack of OpcA. The latter possibility is supported by the observation that there are sequences within *Nostoc* sp. ATCC 29133 which cross-hybridize with an *opcA* probe (Summers *et al.*, 1995b).

Virtually no information regarding the way in which OpcA interacts with G6PDH subunits can be inferred from analysis of the three known *opcA* sequences. The OpcA proteins from *Nostoc* sp. ATCC 29133 (Summers *et al.*, 1995a), *Synechococcus* sp. PCC 7942 (Newman *et al.*, 1995) and *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) exhibit considerable similarities (>65%) to each other, but not to any other known proteins. However, it may be that OpcA represents the first member of a novel class of proteins involved in establishing the quaternary structure of other proteins and in this way would be analogous to the molecular chaperones (for a review see Hendrick & Hartl, 1993) involved in establishing the correct tertiary structure. We are currently investigating the interactions between G6PD subunits and OpcA, and the assembly of oligomeric, catalytically-active G6PDH.

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REFERENCES

- Anderson, S. L. & McIntosh, L. (1991). Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. *J Bacteriol* **173**, 2761–2767.
- Apte, S. K., Rowell, P. & Stewart, W. D. P. (1978). Electron donation to ferredoxin in heterocysts of the N_2 -fixing alga *Anabaena cylindrica*. *Proc R Soc Lond Ser B* **200**, 1–25.
- Astier, C., Elmorjani, K., Meyer, I., Joset, F. & Herdman, M. (1984). Photosynthetic mutants of the cyanobacteria *Synechocystis* sp. strains PCC 6714 and PCC 6803 – para-hydroxymercuribenzoate as a selective agent. *J Bacteriol* **158**, 659–664.
- Carr, N. G. (1973). Metabolic control and autotrophic physiology. In *The Biology of Blue-Green Algae*, pp. 39–65. edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.
- Cossar, J. D., Rowell, P. & Stewart, W. D. P. (1984). Thioredoxin as a modulator of glucose-6-phosphate dehydrogenase in a N_2 -fixing cyanobacterium. *J Gen Microbiol* **130**, 991–998.
- Doolittle, W. F. & Singer, R. A. (1974). Mutational analysis of dark endogenous metabolism in the blue-green bacterium *Anacystis nidulans*. *J Bacteriol* **119**, 677–683.
- Gleason, F. K. (1996). Glucose-6-phosphate dehydrogenase from the cyanobacterium *Anabaena* sp. PCC 7120 – purification and kinetics of redox modulation. *Arch Biochem Biophys* **334**, 277–283.
- Grossman, A. & McGowan, R. E. (1975). Regulation of the glucose-6-phosphate dehydrogenase in blue-green algae. *Plant Physiol* **55**, 658–662.
- Hames, B. D. (1981). An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: a Practical Approach*, pp. 1–86. Edited by B. D. Hames & D. Rickwood. Oxford: IRL Press.
- Hendrick, J. P. & Hartl, F.-U. (1993). Molecular chaperone functions of heat shock proteins. *Annu Rev Biochem* **62**, 349–384.
- Ingram, L. O., Clader, J. A., Van Baalen, C., Plucker, F. E. & Parker, P. L. (1973). Role of reduced exogenous organic compounds in the physiology of the blue-green bacteria (algae): photoheterotrophic growth of a 'heterotrophic' blue-green bacterium. *J Bacteriol* **114**, 695–700.
- Kaneko, T., Sato, S., Kotani, H. & 21 other authors (1996). Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* **3**, 109–136.
- Newman, J., Karakaya, H., Scanlan, D. J. & Mann, N. H. (1995). A comparison of gene organization in the *zwf* region of the genomes of the cyanobacteria *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120. *FEMS Microbiol Lett* **133**, 187–193.
- Pearce, J. & Carr, N. G. (1969). The incorporation and metabolism of glucose by *Anabaena variabilis*. *J Gen Microbiol* **54**, 451–462.
- Pelroy, R. A., Kirk, M. R. & Bassham, J. A. (1976). Photosystem II regulation of macromolecule synthesis in the blue-green alga *Aphanocapsa* 6714. *J Bacteriol* **128**, 623–632.
- Prentki, P. & Krisch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303–313.
- Rippka, R., Deruelles, J., Waterbury, J., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.
- Rowell, P. & Simpson, G. G. (1990). The role of amino acids in the regulation of cyanobacterial glucose-6-phosphate dehydrogenase. In *Inorganic Nitrogen Metabolism in Plants and Microorganisms. Uptake and Metabolism*, pp. 137–143. Edited by W. R. Ullrich, C. Rigano, A. Fuggi & P. J. Aparicio. Berlin & Heidelberg: Springer.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scanlan, D. J., Mann, N. H. & Carr, N. G. (1989). Effect of iron and other nutrient limitations on the pattern of outer membrane proteins in the cyanobacterium *Synechococcus* PCC 7942. *Arch Microbiol* **152**, 224–228.

- Scanlan, D. J., Bloye, S. A., Mann, N. H., Hodgson, D. A. & Carr, N. G. (1990). Construction of *lacZ* promoter probe vectors for use in *Synechococcus*: application to the identification of CO₂-regulated promoters. *Gene* **90**, 43–49.
- Scanlan, D. J., Newman, J., Sebahia, M., Mann, N. H. & Carr, N. G. (1992). Cloning and sequence analysis of the glucose-6-phosphate dehydrogenase gene from the cyanobacterium *Synechococcus* PCC 7942. *Plant Mol Biol* **19**, 877–880.
- Scanlan, D. J., Sundaram, S., Newman, J., Mann, N. H. & Carr, N. G. (1995). Characterization of a *zwf* mutant of *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **177**, 2550–2553.
- Schaeffer, F. & Stanier, R. Y. (1978). Glucose-6-phosphate dehydrogenase of *Anabaena* sp. *Arch Microbiol* **116**, 9–19.
- Silman, N. J., Carr, N. G. & Mann, N. H. (1995). ADP-ribosylation of glutamine synthetase in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **177**, 3527–3533.
- Smith, A. J. (1982). Modes of cyanobacterial carbon metabolism. In *The Biology of Cyanobacteria*, pp. 47–86. Edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.
- Summers, M. L. & Meeks, J. C. (1997). Transcriptional regulation of *zwf*, encoding glucose-6-phosphate dehydrogenase, from the cyanobacterium *Nostoc punctiforme* strain ATCC 29133. *Mol Microbiol* **22**, 473–480.
- Summers, M. L., Meeks, J. C., Chu, S. & Wolf, R. E., Jr (1995a). Nucleotide sequence of an operon in *Nostoc* sp. strain ATCC 29133 encoding four genes of the oxidative pentose phosphate cycle. *Plant Physiol* **107**, 267–268.
- Summers, M. L., Wallis, J. G., Campbell, E. L. & Meeks, J. C. (1995b). Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium *Nostoc* sp. strain ATCC 29133. *J Bacteriol* **177**, 6184–6194.
- Udvardy, J., Borbely, G., Juhasz, A. & Farkas, G. L. (1984). Thioredoxins and the redox modulation of glucose-6-phosphate dehydrogenase in *Anabaena* sp. strain PCC 7120 vegetative cells and heterocysts. *J Bacteriol* **157**, 681–683.
- Wyman, M., Gregory, R. P. F & Carr, N. G. (1985). Novel role of phycoerythrin in a marine cyanobacterium, *Synechococcus* strain DC2. *Science* **230**, 818–820.
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