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 oxygencic photosynthesis and for which phototrophy 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 phototrophic metabolic mode, involving the reductive pentose phosphate (RPP) pathway 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 insertion mutants of the gene (zwf) encoding the key enzyme of the OPP, glucose-6-phosphate dehydrogenase (G6PDH), are considered. Wild-type and zwf insertion 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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(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 phototrophic cyanobacterium, could no longer grow in the dark at the expense of fructose or grow under any trophic cyanobacterium, could no longer grow in the

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 (Scheffer & 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 Amino 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 b_5 and subunit IV of the cytochrome b_5 complex (Newman et al., 1995). There are two PstI sites in pDB: one in the multiple-cloning site and one 100 bp downstream of the opcA gene. Digestion of pDB with PstI yielded two fragments, one of which (48 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 BgIII site approximately in the middle of the opcA gene. Plasmid pHP45Ω carries the 2 kb Ω fragment consisting of the antibiotic resistance gene adaA, confering resistance to streptomycin and spectinomycin, flanked by short inverted repeats carrying transcription and translation termination signals and synthetic polylinkers including BamHI sites (Prentki & Krish, 1984). The Ω fragment was produced by BamHI digestion of pHP45Ω and was inserted into the compatible BgIII 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'-CCCGGATCCTAGAAA-

METHODS

Organisms and growth conditions. The freshwater cyanobacterial strains Anabaena sp. PCC 7120, Synechococcus sp. PCC 7942, Pleistocene boryanum PCC 73110, Synechocystis sp. PCC 6803 and Nostoc sp. MAC PCC 8009 were grown in BG11 and BG11, media (Rippka et al., 1979) as appropriate, under continuous illumination (20 μE m^-2 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 μg ml^-1. Escherichia coli strains were grown in LB medium and 2 × YT medium (Sambrook et al., 1989) as appropriate. Ampicillin and kanamycin were used at 50 and 25 μg ml^-1, respectively.
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 their catalytic activity (M1 < 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 our 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, was 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 stain name; the units (U) 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, Plectonema 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.
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 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...
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 min⁻¹mg⁻¹, which represents 14% of wild-type activity (110.9 nmol NADPH formed min⁻¹mg⁻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 extracts from cultures grown in the presence or absence of exogenous 10 mM 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...
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**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 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 & Harrl, 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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