JOURNAL OF BACTERIOLOGY, July 1997, p. 4513–4522 Vol. 179, No. 14
0021-9193/97/$04.00+0

Functional Complementation of an *Escherichia coli gap* Mutant Supports an Amphibolic Role for NAD(P)-Dependent Glyceraldehyde-3-Phosphate Dehydrogenase of *Synechocystis* sp. Strain PCC 6803

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Received 7 January 1997/Accepted 30 April 1997

The *gap-2* gene, encoding the NAD(P)-dependent D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH2) of the cyanobacterium *Synechocystis* sp. strain PCC 6803, was cloned by functional complementation of an *Escherichia coli gap* mutant with a genomic DNA library; this is the first time that this cloning strategy has been used for a GAPDH involved in photosynthetic carbon assimilation. The *Synechocystis* DNA region able to complement the *E. coli gap* mutant was narrowed down to 3 kb and fully sequenced. A single complete open reading frame of 1,011 bp encoding a protein of 337 amino acids was found and identified as the putative *gap-2* gene identified in the complete genome sequence of this organism. Determination of the transcriptional start point, identification of putative promoter and terminator sites, and orientation of the truncated flanking genes suggested the *gap-2* transcript should be monocystronic, a possibility further confirmed by Northern blot studies. Both natural and recombinant homotetrameric GAPDH2s were purified and found to exhibit virtually identical physicochemical and kinetic properties. The recombinant GAPDH2 showed the dual pyridine nucleotide specificity characteristic of the native cyanobacterial enzyme, and similar ratios of NAD- to NADP-dependent activities were found in cell extracts from *Synechocystis* as well as in those from the complemented *E. coli* clones. The deduced amino acid sequence of *Synechocystis* GAPDH2 presented a high degree of identity with sequences of the chloroplastic NADP-dependent enzymes. In agreement with this result, immunoblot analysis using monospecific antibodies raised against GAPDH2 showed the presence of the 38-kDa GAPDH subunit not only in crude extracts from the *gap-2*-expressing *E. coli* clones and all cyanobacteria that were tested but also in those from eukaryotic microalgae and plants. Western and Northern blot experiments showed that *gap-2* is conspicuously expressed, although at different levels, in *Synechocystis* cells grown in different metabolic regimens, even under chemoheterotrophic conditions. A possible amphibolic role of the cyanobacterial GAPDH2, namely, anabolic for photosynthetic carbon assimilation and catabolic for carbohydrate degradative pathways, is discussed.

Phosphorylating D-glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) are enzymes involved in the central pathways of carbon metabolism that have been found in all organisms studied so far (16). Due to their key metabolic roles, these proteins and their corresponding *gap* genes have been well characterized in many organisms (8, 16). In the case of the widespread glycolytic GAPDH, by far the best-studied member of this enzyme family, the knowledge of its structure has allowed directed mutagenesis of essential amino acid residues of the active site and the eventual detailed description of the enzymatic mechanism (12, 18, 44).

Three distinct GAPDH enzymes with different subcellular localizations and performing diverse roles are present in photosynthetic eukaryotic organisms: (i) a typical NAD-dependent glycolytic enzyme (EC 1.2.1.12), similar to that found in all organisms so far studied and located in the cytoplasm; (ii) the NADP-dependent GAPDH (EC 1.2.1.13), a key component of the reductive pentose phosphate (RPP) cycle, which is located in the chloroplast stroma (7, 42); and (iii) a cytosolic nonphosphorylating NADP-dependent GAPDH (EC 1.2.1.9), characteristic of photosynthetic eukaryotes, that has been proposed to metabolize trioses exported from the chloroplast but whose precise function remains to be established (29, 41). In contrast, a single GAPDH enzyme exhibiting comparable activity levels with NAD or NADP as the cofactor (EC 1.2.1.-) has been found in photoautotrophic cyanobacteria and claimed, on the basis of preliminary biochemical data, to be involved in both photosynthetic (anabolic) and glycolytic (catabolic) carbon metabolism (23). However, recent reports on the existence of different *gap* genes in cyanobacteria (8, 28, 48) raised the question of their functionality and possible physiological roles of their products. On the other hand, the possible existence of a single NAD(P)-dependent GAPDH in the uncompartmented cyanobacterial cell, whose characteristics are yet to be fully described, raised several as yet unanswered questions, such as its involvement in both photosynthetic and degradative carbon metabolism, its regulation at the protein or RNA level, and its relationships with the metabolism of other essential bioelements like N and P. This NAD(P)-dependent enzyme, which we have called GAPDH2, could also provide a good system with which to investigate at the molecular level the basis of NAD or NADP specificity. Furthermore, since the *gap* genes have been extensively used in evolutionary studies (8, 16, 28), an extensive knowledge of the cyanobacterial *gap* genes might

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help to elucidate chloroplast evolution and photosynthesis development.

In this work, the *gap-2* gene encoding GAPDH2, whose anabolic role in photosynthetic carbon assimilation is well established, was cloned by means of functional complementation of an *Escherichia coli gap* mutant from *Synechocystis* sp. strain PCC 6803, a unicellular cyanobacterium able to grow heterotrophically. This cloning strategy, previously used for typical glycolytic *gap* genes (5, 11, 51), has allowed us to show that the cyanobacterial GAPDH2 is able to efficiently perform a catabolic role in vivo, raising the question of whether it is an amphibolic or dual-operating enzyme in *Synechocystis* sp. strain PCC 6803. Despite the finding of two different *gap* genes in the *Synechocystis* sp. strain PCC 6803 genome (25), neither they nor their products have been characterized so far, and a detailed study of their possible regulation by trophic conditions remained to be done. The expression of *gap-2* in *Synechocystis* sp. strain PCC 6803 has been analyzed at both the protein and mRNA levels, and the GAPDH2 has been purified and kinetically characterized. In agreement with the amphibolic character proposed for this protein in *Synechocystis* sp. strain PCC 6803, the *gap-2* product is conspicuously present under all cultural conditions tested, even in heterotrophically grown cells.

MATERIALS AND METHODS

Organisms and growth conditions. *Synechocystis* sp. strain PCC 6803 and the other cyanobacterial strains used in this study (see Table 2) were cultured at 30°C in BG11 medium (34) supplemented with 1 g of sodium bicarbonate per liter and bubbled with 1.5% (vol/vol) CO_2 in air under continuous white light (25 W/m^2). When indicated, the usual nitrogen source ($NaNO_3$, 20 mM) was omitted (N-starved cells) or replaced with 15 mM NH_4Cl buffered with 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)–NaOH (pH 7.5). When glucose was added to the cultures, it was supplied at a final concentration of 10 mM. Cells cultured heterotrophically with glucose were grown in darkness and subjected to a 5-min white light activation period per day, a treatment needed in this case but one that did not promote photosynthetic growth (1). Cells cultured in BG11 medium without sodium phosphate (standard concentration was 0.2 mM) for 4 days were considered phosphate-limited cells (19). After this time, cultures remained viable since growth resumed, after a lag, upon readdition of phosphate. All other cyanobacterial strains were grown photoautotrophically in normal BG11 medium with nitrate as the nitrogen source.

Functional complementation experiments were initially performed with the *E. coli gap* strain DF221 (22), obtained from the *E. coli* Genetic Stock Center (Yale University), which contains an amber mutation in the *gap-1* gene. Another *E. coli gap* mutant, W3CG, in which this gene is inactivated by transposon Tn*10* insertion was later used (18). Both strains were cultured in M63 medium (22) supplemented with succinate (0.4%, wt/vol) and glycerol (0.1%, vol/vol) or, in complementation studies, with glucose (0.4%, wt/vol) and, when necessary, ampicillin (50 μg/ml) and tetracycline (35 μg/ml). *E. coli* K-12 or DH5α was cultured either in M63 plus glucose or in Luria broth (LB) medium, supplemented when necessary with ampicillin (100 μg/ml). M63 plus glucose and LB, in which the *gap* mutants were unable to grow, were used, supplemented with the appropriate antibiotics, as selective media to check for functional complementation. All *E. coli* cells were grown at 37°C with continuous shaking at 200 rpm.

Enzyme assays and protein techniques. All reagents were of analytic grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.). D-Glyceraldehyde-3-phosphate (G3P) dehydrogenase (phosphorylating) activity was determined spectrophotometrically at 30°C by monitoring either the generation (forward direction, oxidation of G3P in the presence of sodium arsenate) or disappearance (reverse reaction, reduction of 1,3-bisphosphoglycerate) of NAD(P)H at 340 nm (29). A coupled assay, in which aldolase (1 U/ml) produced the stoichiometric breakage of fructose-1,6-bisphosphate (1 mM) to G3P and dihydroxyacetone-phosphate, the first product being the actual substrate of the oxidative reaction (40), was usually used during enzyme purification. For kinetic studies, however, DL-G3P in aqueous solution was used at a final 1 mM concentration of the D-isomer. One unit of enzyme was defined as the amount which catalyzes the generation, or disappearance, of 1 μmol of NAD(P)H per min. The esterase activity of GAPDH was determined spectrophotometrically at 30°C by monitoring the *p*-nitrophenol generated in the hydrolysis of *p*-nitrophenylesters at 400 nm (44). All kinetic parameters given represent average values of at least four independent determinations.

Protein concentration was estimated by either the Bradford (4) or the Lowry (27) technique, using ovalbumin as a standard. Electrophoresis of protein extracts was done by the Laemmli method in 12% (wt/vol) acrylamide slab gels in the presence of sodium dodecyl sulfate (SDS) or under nondenaturing conditions in polyacrylamide gradient gels (7.5 to 15% [wt/vol] acrylamide/0 to 30% [vol/vol] glycerol), using a Miniprotean II apparatus (Bio-Rad, Richmond, Calif.) and Sigma MW-SDS-701 or MW-ND-500 protein markers, respectively. Nondenaturing gels were stained for NAD- or NADP-linked GAPDH activity in situ by incubation for 30 min in the standard reaction mixture for the oxidative reaction, with either NAD or NADP, supplemented with 20 μM phenazine methosulfonate and 1 mM *p*-nitroblue tetrazolium chloride. The enzyme band was located by the appearance of a deep-purple band of insoluble formazan. In the absence of aldolase or fructose 1,6-diphosphate (and therefore G3P) or arsenate in the staining system, no color was developed. Isoelectric focusing (35) was done with the same electrophoretic system in 5% (wt/vol) acrylamide slab gels holding ampholite-generated pH gradients (pH range, 3.5 to 10.0; Pharmalite 3.5-10; Pharmacia Biotech, Uppsala, Sweden), 25 mM NaOH and 20 mM HCl as cathode and anode solutions, respectively, and the Sigma 3.6-9.3 IEF-MIX isoelectric focusing protein marker kit. Analytical fast protein liquid chromatography (FPLC; Pharmacia Biotech) gel filtration of purified GAPDH2 preparations was performed by isocratic elution on a Superose 6HR 10/30 column (1 by 30 cm), using an automated Pharmacia FPLC system. All protein physicochemical parameters presented are average values of three independent determinations.

For amino-terminal protein sequencing, 50 pmol of pure protein, from either the recombinant or native GAPDH2, was resuspended in water and blotted to polyvinylidenedifluoride filters (Millipore, Bedford, Mass.). The samples were then subjected to microsequencing by automated Edman degradation on a model 476A protein sequencer (version 2.00; Applied Biosystems, Foster City, Calif.), and 10 amino acids from the amino-terminal end were sequenced.

Cloning strategy and DNA manipulation. Restriction and modifying enzymes were purchased from Pharmacia Biotech, Promega (Madison, Wis.), or Boehringer Mannheim (Mannheim, Germany).

Competent cells of *E. coli* DF221 were transformed with a total genomic DNA library from *Synechocystis* sp. strain PCC 6803 cloned in the *Cla*I site (insert sizes in the range of 6 to 10 kb) of plasmid pBluescript SK$^+$ (pBS); Stratagene, La Jolla, Calif.). The transformation mixture was cultured in LB liquid medium supplemented with 50 μg of ampicillin per ml. If growth took place in the selective medium, cells were harvested by centrifugation, and an alkaline extraction of plasmid was done (36). The plasmid was transferred into *E. coli* DH5α to avoid potential DNA rearrangements that may occur in strain DF221. For further expression experiments, and since the rate of spontaneous reversion of the amber *gap* mutant was rather high (ca. 10^{-3}), the *gap E. coli* mutant strain W3CG (18) was chosen. The *gap-1* gene of *Anabaena* sp. strain PCC 7120 was cloned by using the same functional complementation strategy, overexpression of the cyanobacterial NAD-specific GAPDH1 being found in the recombinant *E. coli* clone (49).

Chromosomal DNA was isolated from cyanobacterial cells as previously described (6). For DNA-DNA hybridization experiments (Southern blotting), the method of Ausubel et al. (2) was used. Samples of cyanobacterial genomic DNA were completely digested with different restriction enzymes, run in 0.7% (wt/vol) agarose gels, and blotted to nylon membranes (Zetaprobe; Bio-Rad, Richmond, Calif.). The filter was then prehybridized in a buffer (5× SSC [1× SSC is 0.18 M NaCl plus 10 mM sodium citrate {pH 7.0}], 5× Denhardt's solution [1× Denhardt's solution is 0.02% {wt/vol} Ficoll, 0.02% {wt/vol} polyvinylpyrrolidone, and 0.02% {wt/vol} bovine serum albumin]) containing 50 μg of salmon sperm DNA per ml and 0.1% (wt/vol) SDS. Either an *Ase*I restriction fragment of ca. 2.1 kb from the pFV8 plasmid insert, an internal *Bgl*II-*Ava*I fragment of *gap-2*, or a PCR-generated *gap-2* fragment of ca. 0.94 kb containing 93% of the coding sequence, obtained from genomic DNA by using degenerate oligonucleotides based on highly conserved regions of GAPDHs (16) [sense primer, 5′-AAT(C)GGA(CGT)TTC(T)GGA(CGT)A(C)GA(G)ATA(CT)GGA(CGT)A(C)G-3′; antisense primer, 5′-ACCATG(A)CTG(A)TTG(A)CTC(T)ACC(G)CC-3′], was used as a probe after radiolabelling with ^{32}P (Random-Primer kit; Boehringer Mannheim). Homologous hybridization was performed at 65°C followed by sequential washing of the filter at room temperature with 2× SSC–0.1% (wt/vol) SDS for 5 min, 2× SSC–0.1% (wt/vol) SDS for 15 min, 0.5× SSC–0.1% (wt/vol) SDS for 15 min, 0.1× SSC–0.1% (wt/vol) SDS for 15 min, and finally 0.1× SSC–1% (wt/vol) SDS for 30 min at 42°C. In heterologous conditions, the hybridization temperature was 55°C and the final highly stringent washing step was omitted. The nylon filters were then exposed to films (Kodak X-100) at −80°C and eventually developed.

For DNA sequencing, plasmid pFV8 was subcloned by sequential exonuclease deletions (Erase a Base kit; Promega), and resulting plasmids were then sequenced in both strands by a modification of the dideoxy-chain termination method (37) with [^{35}S]dATP and *Taq* polymerase (Taq Track kit; Promega) as instructed by the manufacturer.

For total RNA extraction, a modification of the one-step procedure of Chomczynski and Sacchi (10) was used as described by the manufacturer (RNAgents Total RNA Isolation System; Promega) with cells from *Synechocystis* sp. strain PCC 6803 grown under different culture conditions until mid-exponential phase. Separation of RNA on formaldehyde-agarose gels and hybridization to nylon membranes (Zeta-Probe; Bio-Rad) were carried out according to the manufacturer's instructions; 15 μg of total RNA was loaded per lane. The hybridization and washing steps (with SSP [1× SSP is 0.18 M NaCl plus 10 mM $NaH_2PO_4 \cdot H_2O$; pH 7.4] solution instead of SSC) were those described for the Southern blot protocol

using either the same probes or another one from *rnpB* corresponding to the RNA part of the *Synechocystis* sp. strain PCC 6803 RNase P ribozyme (50) as a loading control. The filters were then applied to an InstantImager Electronic Autoradiography apparatus (Packard Instrument Co., Downers Grove, Ill.), and the resulting radioactivity per mark was counted and analyzed.

Primer extension analysis was done with 10 μg of total RNA and a 23-nucleotide-long oligonucleotide primer from a sequence of the 5′ region of the coding sequence, reading upstream from the complementary chain (5′-CGTCC GATCCGTCCAAATCCGTT-3′). Avian myeloblastosis virus reverse transcriptase (Promega) was used. Deoxynucleotides, including [α-^{32}P]dCTP (3,000 Ci/mmol), were added at 1 mM (final concentration) in the reaction mixture (30 min, 42°C), so that the final cDNA fragment was thoroughly radiolabelled. RNA was then totally digested by RNase treatment (30 min, 37°C), and the resulting preparation was applied to a Sephacryl S-400 minicolumn (Pharmacia Biotech). A standard sequencing reaction of the insert of pFV8, using as the primer the same oligonucleotide, was run and electrophoresed parallel to the radiolabelled cDNA extension product.

Purification of natural and recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2. *Synechocystis* sp. strain PCC 6803 cells from late logarithmic growth phase were harvested by centrifugation at 10,000 × *g* in a Sorvall RC5C centrifuge at 4°C and washed twice with 50 mM Tris-HCl (pH 7.5) buffer, and the cell pellet was stored at −20°C. Frozen cells were resuspended in 50 mM Tris-HCl (pH 7.5) buffer with 10% (vol/vol) glycerol, 10 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, and 5 mM potassium phosphate (standard buffer) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), ε-aminocaproic acid (2 mM), and benzamidine (0.5 mM), at a ratio of 3 ml/g (fresh weight) of cells. The cells were then disrupted by ultrasonic treatment in a chilling water bath, using a Branson 25U Sonifier at medium strength (six 30-s pulses with intermediate 30-s periods of rest). The resulting broken-cell suspension was then supplemented with $MgCl_2$ up to 10 mM and centrifuged at 40,000 × *g* for 30 min. This greenish supernatant (considered the crude extract) was treated with 10 mM streptomycin sulfate for 1 h at 4°C under continuous stirring and centrifuged as described above, and the resulting supernatant was treated with solid ammonium sulfate; the protein fraction obtained by 40 to 80% ammonium sulfate saturation was collected. This protein pellet was resuspended in a minimal volume (ca. 10 ml) of standard buffer, dialyzed overnight against 5 liters of 20 mM Tris-HCl buffer (pH 7.5) with the same additions as the standard buffer except that 0.1 mM DTT was used and no potassium phosphate was added (dialysis buffer), and then applied at a flux of 30 ml/h to a DEAE-cellulose DE-52 (Whatman, Maidstone, England) column (3 by 12 cm) preequilibrated with standard buffer. After a thorough wash, anion-exchange chromatography was performed with a linear gradient of potassium phosphate (5 to 200 mM, pH 7.5; total volume, 300 ml) in standard buffer. Fractions of 4 ml were collected, and those which showed enzymatic activity were pooled. Solid ammonium sulfate was added up to 25% (wt/vol) to the resulting enzyme preparation, which was applied at low flux (12 ml/h) to a phenyl-Sepharose HP (Pharmacia Biotech) column (1.6 by 18 cm) preequilibrated with standard buffer with 25% (wt/vol) ammonium sulfate. After thorough washing of the column, the hydrophobic interaction chromatography was developed by a double linear gradient (total volume, 150 ml), with decreasing ammonium sulfate (from 25 to 0%, wt/vol) and increasing glycerol (from 10 to 20%, vol/vol) in standard buffer. The 1.9-ml fractions of the single activity peak were pooled, dialyzed for 3 h against dialysis buffer, and then introduced at a flux of 3 ml/h in a Blue Sepharose CL-4B (Pharmacia Biotech) column (1 by 5 cm) previously equilibrated with dialysis buffer. After thorough washing of the column with this buffer, the pH was raised to 8.5 and the potassium phosphate concentration was increased to 50 mM. After a wash with two column volumes of this buffer, the enzyme activity was eluted with a 5-ml pulse of 10 mM NAD or NADP in dialysis buffer, and fractions with maximal activity were collected and pooled. This purified GAPDH preparation showed only one 38-kDa protein band in SDS-polyacrylamide minigels. Cell extracts of other cyanobacteria were obtained by using the procedure described above for *Synechocystis* sp. strain PCC 6803 cells.

A similar procedure was used for purification of recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 from *E. coli* W3CG *gap* mutant strain cells transformed with pFV8 and cultured in LB medium supplemented with ampicillin (100 μg/ml) and tetracycline (35 μg/ml). Late-logarithmic-phase cells from 2 liters of medium were collected as described above, and the same purification procedure was followed. In this case, the anion-exchange chromatography was essential in order to separate the enzyme from the great amount of proteins and lipids present in the *E. coli* crude extracts.

Column chromatofocusing was used as both an analytical and a preparative technique to characterize the purified enzyme preparations and check for the possible presence of isoforms. A column bed of Polybuffer exchanger PBE (1 by 18 cm) and a solution of Polybuffer 74-HCl (1:10 dilution, adjusted to pH 3.5) were used. The activity was eluted as instructed by the manufacturer (Pharmacia Biotech) by the continuous pH gradient (from 7.5 to 3.5) generated by washing the column with the Polybuffer solution.

Immunological techniques. A rabbit was injected with 500 μg of pure GAPDH2 protein in aqueous solution 1:1 with Freund's coadjuvant. After 21 days, a small sample of blood was collected, and a second dose of 500 μg of protein was injected (20). After 2 weeks, 50 ml of rabbit blood was collected and serum was separated by letting it coagulate overnight at 4°C and then centrifuging it. The obtained serum, containing monospecific anti-GAPDH2 polyclonal antibodies, was sampled and stored at −20°C.

Immunoblot (Western blot) assays of protein samples were carried out after electrophoresis in SDS–12% polyacrylamide slab gels. Proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad) by using a Biometra Fast-Blot B32 cell (B. Braun Biotech, Melsungen, Germany) and incubated with 1:250-fold diluted antiserum in Tris-buffered saline (TBS) with 5% (wt/vol) skim milk. The membrane was then washed four times (15 min each) in TBS plus 0.05% (vol/vol) Tween 20 (TBSt) and incubated for 2 h with a goat anti-rabbit immunoglobulin G antibody-peroxidase conjugate (1:1,000; Boehringer Mannheim). After four 15-min rounds of washing with TBSt, the nitrocellulose filter was developed under a mixture of TBS, 2 mM H_2O_2, and 10 mM 4-chloro-3-naphthol in methanol. Filters were processed and when necessary quantified with an analytical imaging instrument (Bio Image; Millipore).

Nucleotide and amino-terminal protein sequence accession numbers. The EMBL/GenBank database accession number for the *Synechocystis* sp. strain PCC 6803 DNA fragment containing the *gap-2* gene is X83564. The accession number in the SwissProt database for both natural and recombinant GAPDH2 amino-terminal protein sequences is P80506.

TABLE 1. NAD(P)-dependent GAPDH specific activities of cell extracts of *Synechocystis* sp. strain PCC 6803 and different *E. coli* strains and growth ability of *E. coli* in different media

Organism	GAPDH sp act (U/mg of protein)[a]		Growth on M63 supplemented with[b]:	
	NAD dependent	NADP dependent	Succinate plus glycerol	Glucose
Synechocystis sp. strain PCC 6803	0.048	0.049	NA	NA
E. coli K-12 (wild type)	0.860	<0.001[c]	+++	+++
E. coli DF221 (*gap*, Am mutation)	<0.001	<0.001	+	−
E. coli W3CG (*gap gap-1*::Tn*10* Tc^r)	<0.001	<0.001	++	−
E. coli DF221(pFV8)	0.601	0.610	+	++
E. coli W3CG(pFV8)	6.602	6.525	++	+++

[a] *E. coli* activity was determined in glucose-grown cultures except for *gap* mutants. Means of at least three different experiments are presented; the standard error was less than 15% in all cases.

[b] Only for *E. coli* strains. Growth phenotypes were scored as follows: +++, rapid growth (~75-min doubling time); ++, moderate growth (~105-min doubling time); +, slow growth (~140-min doubling time); −, no detectable growth. NA, not applicable.

[c] Negligible values (below the detection limit).

RESULTS AND DISCUSSION

Functional complementation of a *gap E. coli* mutant by the *gap-2* gene of *Synechocystis* sp. strain PCC 6803. Plasmids containing a total genomic DNA library of *Synechocystis* sp. strain PCC 6803 constructed on the *Cla*I site of the pBS SK$^+$ vector were used to transform the *gap E. coli* mutant DF221. Plasmid extraction from clones grown in liquid LB medium supplied with ampicillin indicated that a unique 10.6-kb plasmid (pFV1) was present in the complemented mutant. pFV1 was subcloned until a plasmid of ca. 5.9 kb containing a 3-kb *Kpn*I-*Dra*I insert (pFV8), still retaining the capacity to complement the *gap* mutation, was constructed. As shown in Table 1, whereas the NAD-dependent activity of the typical glycolytic enzyme was found in crude extracts of wild-type *E. coli* strains like K-12, no significant GAPDH activity was detected in the *gap E. coli* mutants. In contrast, crude extracts from the complemented *gap* mutants harboring plasmid pFV8 also exhibited significant levels of NADP-dependent activity, the values obtained with both pyridine nucleotides being similar, a distinctive feature of *Synechocystis* sp. strain PCC 6803 cell extracts. These results strongly suggested that the cyanobacterial NAD(P)-dependent GAPDH2, and not the strictly NAD-dependent glycolytic

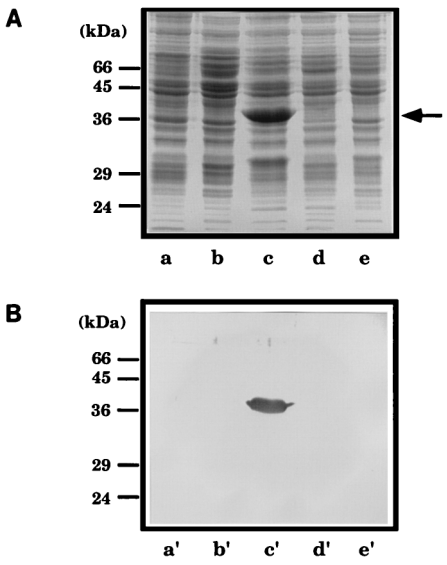

FIG. 1. (A) Coomassie blue-stained SDS-PAGE electrophoretogram showing the protein patterns of cell extracts from several *E. coli* strains grown in different media. Lane a, wild-type K-12 strain in minimal medium M63 plus glucose; lane b, DF221 *gap* mutant in M63 plus succinate and glycerol; lane c, W3CG *gap* mutant transformed with pFV8 plasmid in M63 plus glucose and ampicillin; lane d, W3CG mutant in M63 plus succinate and glycerol; lane e, W3CG mutant transformed with plasmid pBS SK^+ in M63 plus succinate, glycerol, and ampicillin. The arrow marks the position of the overexpressed 38-kDa recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 protein in the cell extract corresponding to lane c. (B) Immunoblot analysis with monospecific antibodies raised against *Synechocystis* sp. strain PCC 6803 GAPDH2 of the cell extracts used for panel A. About 70 μg of protein was loaded per lane in all cases. The positions and molecular masses of marker proteins are indicated.

GADPH of the enterobacterium, was the functionally expressed enzyme in the recombinant clones. The growing capacity in LB medium of the *E. coli* W3CG strain harboring plasmid pFV8 was similar to that of wild-type strain K-12 or DH5α (Table 1), suggesting an efficient performance of the *Synechocystis* sp. strain PCC 6803 enzyme in degradative carbon metabolism of the enterobacterium. The finding that cell extracts of the recombinant clones exhibited with both pyridine nucleotide cofactors specific activity levels 10- to 100-fold higher than those found in *Synechocystis* sp. strain PCC 6803 indicated that GAPDH2 was overexpressed. Likewise, whereas no band clearly attributable to the overexpressed GAPDH subunit was detected upon SDS-polyacrylamide gel electrophoresis (PAGE) of total protein preparations of the W3CG mutant, a prominent protein band of ca. 38 kDa could be seen in extracts from the complemented clones (Fig. 1A), its apparent size being slightly greater than that of the typical glycolytic enzyme subunit (36 kDa) but fitting well with that of the purified *Synechocystis* sp. strain PCC 6803 GAPDH2 (see below). The recombinant GAPDH2 subunit was also immunodetected in Western blot analysis of protein extracts from the recombinant *E. coli* clones, using these anti-GAPDH2 antibodies (Fig. 1B), thus demonstrating unambiguously the heterologous overexpression of the *Synechocystis* sp. strain PCC 6803 enzyme.

The functional complementation of *E. coli gap* mutants, widely applied to clone *gap* genes coding for NAD-dependent glycolytic GAPDHs from heterotrophic eubacteria (4, 11, 51), has been successfully used to clone a *Synechocystis* sp. strain PCC 6803 *gap-2* gene involved in photosynthetic carbon assimilation. This suggests that *Synechocystis* sp. strain PCC 6803 GAPDH2 should be a bifunctional enzyme that, in addition to operating in the RPP pathway in photosynthesis, also functions efficiently in glycolysis, the main physiological role for GAPDH in heterotrophic eubacteria. Therefore, the potential products of the *gapC*-like gene *gap-1* or *gap-3*, recently found in some cyanobacterial genomes (8, 28), could in principle not be necessary to fulfill the metabolic demands for a catabolic NAD-dependent GAPDH in these photosynthetic prokaryotes. In fact, *Synechocystis* sp. strain PCC 6803 GAPDH2 is detected and *gap-2* is expressed at high levels under all metabolical regimens tested by us (see below), while the other possible *gap* product in this cyanobacterium, i.e., that of *gap-1*, is either barely detectable or not observed at all (49). Additional evidence suggesting that GAPDH2 should be the only functional GAPDH was obtained from immunotitration experiments with the anti-GAPDH2 antibody, since complete inhibition of both NAD- and NADP-dependent activities was achieved in total protein extracts from *Synechocystis* sp. strain PCC 6803 (see below).

The whole *Synechocystis* sp. strain PCC 6803 DNA insert contained in plasmid pFV8 (a total of 2,984 bp) was completely sequenced in both strands. Since only one complete open reading frame (ORF) of 1,011 bp, around the size expected for a *gap* gene, was found in the insert, we presumed that it corresponded to the *gap-2* gene coding for *Synechocystis* sp. strain PCC 6803 GAPDH2. This was later confirmed by a similarity search using the FASTA program (18a) against the recently published entire genomic sequence of *Synechocystis* sp. strain PCC 6803 (25). The *gap-2* coding sequence found in the pFV8 insert showed 99.5% identity with the ORF sll1342 (positions 3279135 to 3278122) assigned to a *gap-2* gene (25), thus experimentally validating this initial assignment. The 337-amino-acid (aa) predicted GAPDH2 proteins were virtually identical (100% similarity) since the five observed nucleotide changes produced either silent or conservative amino acid changes (two G for A and one I for F). Nucleotide sequences of the flanking regions of *gap-2* in the pFV8 insert were also consistent with the context of this gene in the *Synechocystis* sp. strain PCC 6803 genome: (i) a truncated (1,376-bp) ORF starting 199 bp upstream *gap-2* showed 99.5% identity to the 5′ region of *ape-2* (sll1343), a gene potentially coding an 869-aa aminopeptidase, and (ii) a noncoding 255-bp sequence separated the 5′ end of *gap-2* from another truncated ORF (145 bp) located in the complementary strand, which was 100% identical to the 5′ region of the gene (*murC*, slr1423) likely encoding a 505-aa *N*-acetylmuramatealanine ligase (25). The start codon of *gap-2* was identified as GTG, a fairly common feature of *Synechocystis* sp. strain PCC 6803 genes (25); this identification was later confirmed by direct analysis of the amino-terminal sequence of the GAPDH2 protein (see below). A potential Shine-Dalgarno sequence (5′-GAGG-3′) was found 6 bp upstream of the *gap-2* initiation codon. The transcriptional start point was determined by primer extension analysis of the upstream region of *gap-2* (Fig. 2) to be an A located 48 bp upstream of the initiation codon; 10 bp upstream, a 5′-TATG AT-3′ sequence that could act as a putative −10 region was found. No further upstream −35 region seems to exist, as is common among cyanobacterial genes (14). The noncoding 199-bp downstream region of *gap-2* contained an inverted repeat followed by an oligo(T) tract found 74 bp from the translational termination codon TAG. The ΔG of the potential stem-loop structure formed by this inverted repeat, calculated by the method of Freier et al. (17), is −42.2 kJ/mol. These features are suggestive of a factor-independent eubacterial transcription terminator. Overall, these data suggested that the transcript of *Synechocystis* sp. strain PCC 6803 *gap-2* should be

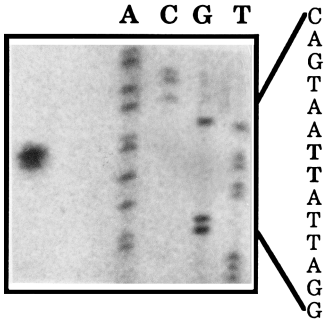

FIG. 2. Mapping of the GAPDH2 presumed transcriptional start point by primer extension analysis of total RNA purified from photoautrophically grown *Synechocystis* sp. strain PCC 6803 cells. The extension products were loaded into the lane on the left. The DNA sequence at the right corresponds to the region of the template strand of *gap-2* around the two T bases (boldface) identified as transcription start sites.

monocistronic and have a size of ca. 1.2 kb, as was further demonstrated by Northern blot analysis (see below). The coding sequence of *gap-2* was inversely oriented with respect to the vector promoter in the insert of plasmid pFV8 and should therefore be transcribed from its own promoter elements in the complemented *E. coli* clones. It should be noted that the other *gap* gene found in the *Synechocystis* sp. strain PCC 6803 genome (*gap-1*, which belongs to the highly conserved *gapC* superfamily [28]) is located in a completely different region of the *Synechocystis* sp. strain PCC 6803 genome (slr0884; positions 1147034 to 1148098) (25). Its deduced amino acid sequence has only about 50% identity with the predicted sequence of GAPDH2 of *Synechocystis* sp. strain PCC 6803.

The nucleotide sequence of *Synechocystis* sp. strain PCC 6803 *gap-2* has high identity, in the range of 70 to 80%, with two other cyanobacterial *gap-2* genes, those of the cyanobacteria *Anabaena variabilis* ATCC 29413 (28) and *Synechococcus* sp. strain PCC 7942 (48) recently reported, and with *gapA* and *-B* genes from higher plants (8, 42) but only about 50% identity with the *gapC*-like genes *gap-1* and *gap-3* from those cyanobacteria (28) as well as with typical glycolytic *gapC* genes from other eubacteria, plants, and animals (16). The deduced amino acid sequence of the *Synechocystis* sp. strain PCC 6803 GAPDH2 displayed high identity (ca. 80%) with those of the chloroplastic NADP-dependent GAPDHs encoded by *gapA* and *-B* genes in higher plants (8, 42) and *gapA* genes in eukaryotic algae (8, 26, 28). Comparison with the amino acid sequences deduced from the different *gap* genes recently reported for other cyanobacteria, namely, those of *A. variabilis* (28) and *Synechococcus* sp. strain PCC 7942 (48), showed high identity (ca. 75%) with those of the *gap-2* genes and much lower identity with the *gapC*-like genes *gap-1* and *gap-3* (60 and 45%, respectively). The overall identities with the deduced amino acid sequences of glycolytic GAPDHs from other eubacteria, plants, or animals were around 50%. It should be noted that the amino-terminal amino acid sequence determined by the Edman degradation method for GAPDH2 purified from *Synechocystis* sp. strain PCC 6803 cells (see below) matches well with the sequence deduced from the cloned *Synechocystis* sp. strain PCC 6803 *gap-2* gene and is similar to that reported for the *Synechococcus* sp. strain PCC 7942 GAPDH2 protein (48). The high degree of identity of the *gap-2* gene cloned by functional complementation with *gapA* and *gapB*, which code for plant chloroplastic GAPDHs, is consistent with the role of its product in photosynthetic carbon assimilation in *Synechocystis* sp. strain PCC 6803.

The amino acid sequence deduced from the *gap-2* gene of *Synechocystis* sp. strain PCC 6803 has been analyzed and found to have the structural features typical of other *gap* genes, including (i) the well-conserved sequence of the active site ^{152}ASCTTNCL^{159} (according to the numbering of the predicted *Synechocystis* sp. strain PCC 6803 GAPDH2) around the C^{154} (in boldface) that forms the thioester bond with the substrate in the catalysis (30) and (ii) the so-called S-loop region (residues 183 to 205) which is involved in the interaction between subunits and the binding of the nucleotide cofactor and displayed prokaryotic signatures (16). A proline residue of this region present in glycolytic GAPDHs, P^{188} according to the numbering of *Bacillus stearothermophilus* GAPDH (5), has been postulated to be involved in pyridine nucleotide cofactor specificity conferring absolute specificity for NAD (12). Noteworthy, this residue is replaced with a serine, S^{193}, in the *Synechocystis* sp. strain PCC 6803 GAPDH2 amino acid sequence; thus, the enzyme is able to use NADP as a cofactor, as is also the case for all *gapA*/*B*-encoded chloroplastic GADPHs so far studied (7, 16). Since, in contrast with these enzymes, the cyanobacterial GAPDH2 is able to use both pyridine nucleotides efficiently (chloroplastic GAPDHs have only marginal activity with NAD), other amino acid residues would probably be involved in the degree of NADP/NAD discrimination by the GAPDHs implicated in photosynthetic carbon assimilation in different organisms. The GAPDH2 from *Synechocystis* sp. strain PCC 6803 has five C residues, two of which (C^{154} and C^{158}, as described above) are clustered in a region directly involved in catalysis (30); the others (C^{19}, C^{75}, and C^{292}) are spread over the protein sequence. These three C residues are, however, probably not involved in reductive enzyme activation since it has been reported that the NADP-dependent GAPDH activity of *Synechocystis* sp. strain PCC 6803 cell extracts is not activated either in vivo by light or in vitro by thiol-reducing reagents like DTT (31, 47), as we have confirmed for both crude and pure preparations of native and recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 (data not shown). All other catalytic residues proposed to be involved in the GAPDH enzymatic mechanism (30) (H^{181}, T^{184}, and R^{234}) are also present in the amino acid sequence deduced from *Synechocystis* sp. strain PCC 6803 *gap-2*.

Properties of purified natural and recombinant GAPDH2 of *Synechocystis* sp. strain PCC 6803. The NAD(P)-dependent GAPDH2 has been purified about 2,000-fold to electrophoretic homogeneity from photoautotrophically grown *Synechocystis* sp. strain PCC 6803 cells with a yield of ca. 30%, using a procedure involving conventional ammonium sulfate fractionation, anion-exchange chromatography, hydrophobic chromatography, and dye-ligand chromatography. The protein analysis by SDS-PAGE of the preparations obtained after the different purification steps demonstrated the homogeneity of the final GAPDH2 preparation, which exhibited high specific activity values, typically ca. 120 U/mg. A single protein band with a molecular mass of ca. 38 kDa was seen in the electrophoretogram of the final enzyme preparation (Fig. 3B). The recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 was also purified to electrophoretic homogeneity (about 30-fold) from *E. coli* W3CG cells harboring pFV8 by using a purification procedure similar to that used for the natural enzyme. A single protein band of 38 kDa, indistinguishable from that of the natural GAPDH2 and with a subunit size slightly greater than that of the typical glycolytic GAPDH, was also found in the final preparation on SDS-polyacrylamide gels (Fig. 4A). It should be noted that both NAD- and NADP-linked activities were monitored throughout the complete purification of natural and recombinant *Synechocystis* sp. strain PCC 6803

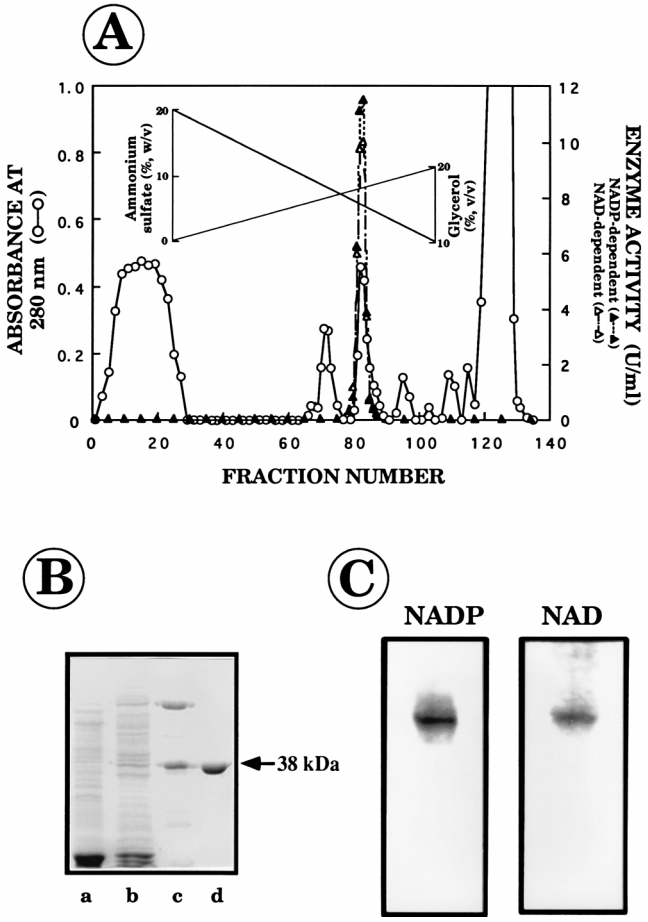

FIG. 3. (A) Protein and G3P dehydrogenase activity elution by a double linear gradient of decreasing ammonium sulfate concentration and increasing concentration of glycerol during hydrophobic interaction chromatography on phenyl-Sepharose HP, a purification step of GAPDH2 from photoautotrophically grown *Synechocystis* sp. strain PCC 6803 cells. Both NAD- and NADP-dependent activities eluted simultaneously as perfectly overlapped symmetrical peaks. (B) Coomassie blue-stained SDS-PAGE electrophoretogram showing different *Synechocystis* sp. strain PCC 6803 GAPDH2 purification step fractions. Lane a, 40 to 88% ammonium sulfate protein fraction; lane b, anion-exchange chromatography eluate pool; lane c, hydrophobic-interaction chromatography eluate pool; lane d, pure protein preparation after dye-ligand chromatography. Aliquots of protein preparations containing either 50 μg (lanes a and b) or 20 μg (lanes c and d) of protein were applied. The arrow points to the 38-kDa GAPDH2 subunit band. (C) Localization, with either NADP or NAD, of G3P dehydrogenase activity of purified natural *Synechocystis* sp. strain PCC 6803 GAPDH2 after PAGE under nondenaturing conditions. Activity staining was performed in situ in the glycerol-polyacrylamide gradient gels incubated with reaction mixtures containing either NADP (left) or NAD (right) and developed with *p*-nitroblue tetrazolium chloride.

GAPDH2, and a perfect overlapping as a single symmetrical peak was observed during all chromatographic steps (Fig. 3A).

We compared purified natural and recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 with regard to various structural and kinetic properties and found no significant differences between them. Thus, they had an homotetrameric structure in the native state as suggested by the virtually identical molecular masses, ca. 160 ± 5 kDa, determined by FPLC gel filtration. Moreover, the two enzymes exhibited the same behavior in nondenaturing glycerol-polyacrylamide gradient gels (estimated molecular mass by this method, ca. 155 ± 5 kDa) as well as the same pI value (ca. 4.85 ± 0.1) upon either column chromatofocusing or isoelectric focusing, indicating identical surface charge distribution and therefore no significant conformational differences between them. The natural and recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2s were also located in the nondenaturing polyacrylamide gels by in situ staining for dehydrogenase activity using tetrazolium dyes with either NAD or NADP as the cofactor; the activity bands showed similar migration patterns (Fig. 3C). The amino-terminal ends of both natural (TRVAINGFGR) and recombinant (MTRVAINGFGR) *Synechocystis* sp. strain PCC 6803 GAPDH2s were sequenced by the Edman degradation method. The virtual identity of the obtained amino acid sequences suggested no differences in primary protein structure between them. However, it is noteworthy that while in the natural enzyme purified from the cyanobacterium the initial methionine residue was removed, the recombinant protein still retained the starting amino acid deduced from the *gap-2* gene. This could be due to a less efficient maturation mechanism in the *E. coli* host or to an excess of protein product for which complete maturation had not been possible.

Both purified natural and recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2s were able to use NAD or NADP with virtually the same efficiency; specific activities of ca. 120 U/mg, similar to those of other GAPDHs (7, 16), were found in the purified preparations. The optimal pH value for the oxidative reaction was in both cases 8.2 ± 0.1, with considerable activity in the range of 7 to 10, and the apparent optimal temperature was 35 ± 2°C. Apparent K_m and V_{max} were measured for both NAD and NADP in this reaction, and identical

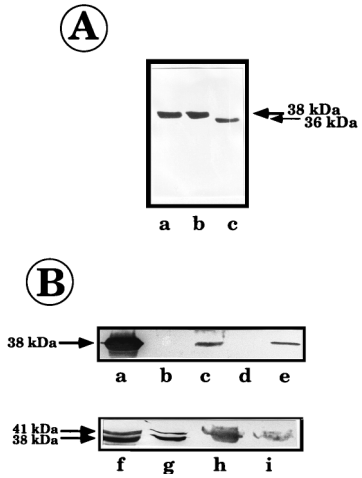

FIG. 4. (A) Coomassie blue-stained SDS-PAGE electrophoretogram showing purified preparations of different GAPDHs. Lane a, purified natural *Synechocystis* sp. strain PCC 6803 GAPDH2 protein; lane b, recombinant *Synechocystis* sp. strain PCC 6803 GAPDH2 protein purified from pFV8-transformed W3CG *gap* mutant; lane c, a typical glycolytic GAPDH from mammalian tissue (skeletal muscle of *Jaculus orientalis*). Aliquots of protein preparations containing 10 μg of purified enzymes were applied. The thick arrow marks the position of the 38-kDa GAPDH2 subunit. Note the lower molecular mass (36 kDa) of the mammalian glycolytic GAPDH subunit (thin arrow). (B) Immunoblot analysis of cell-free protein extracts from different organisms using monospecific antibodies raised against *Synechocystis* sp. strain PCC 6803 GAPDH2. Lane a, *E. coli* W3CG transformed with pFV8; lane b, wild-type *E. coli* strain K-12; lane c, *Synechocystis* sp. strain PCC 6803; lane d, *E. coli* W3CG bearing a pBS SK$^+$ derivative plasmid containing the *gap-1* gene from *Anabaena* sp. strain PCC 7120 and overexpressing GAPDH1; lane e, *Anabaena* sp. strain PCC 7120; lane f, *Antirrhinum majus*; lane g, *Spinacea oleracea*; lane h, *Chlamydomonas reinhardtii* 6145c (a chlorophycean microalga); lane i, *Porphyridium purpureum* SAG 1380-1a (a rhodophycean microalga). About 70 μg of total protein was loaded per lane. Note that anti-GAPDH2 antibodies recognized in higher plant extracts both *gapA* and *gapB* protein products (ca. 38 and 41 kDa, respectively) which constitute the heterotetrameric chloroplastic GAPDHs of these organisms (lanes f and g).

values within the experimental errors (30 ± 5 μM and 7 ± 1 μmol/min, respectively) were estimated for both coenzymes, using the natural and recombinant GAPDH2s. These values are, moreover, very similar to those described for other cytosolic and chloroplastic GAPDHs (7), a characteristic of these enzymes being a typical substrate inhibition, which we took into consideration when calculating the apparent kinetic parameters for pyridine nucleotides. The kinetic parameters of G3P estimated for both proteins were also alike with either NAD or NADP as the cofactor (K_m, 270 ± 30 μM; V_{max}, 17 ± 2 μmol/min), again with virtually no differences between recombinant and natural GAPDHs, clearly indicating that the two should be functionally identical. Both enzymes were also very specific for the phosphorylated substrates, no significant activity being observed with other phosphorylated or nonphosphorylated trioses, like dihydroxyacetone-phosphate, even in the millimolar concentration range (data not shown). On the other hand, the apparent K_m values estimated for NADPH and NADH in the reductive reaction (formation of G3P from 1,3-bisphosphoglycerate) were also virtually identical, 10 ± 1 μM, but the V_{max} values were different, 2.2 ± 0.3 μmol/min for NADPH and 4.5 ± 0.4 μmol/min for NADH, indicating a higher catalytic efficiency for NADH than for NADPH. In fact, this is the only kinetic difference found between both pyridine nucleotides in *Synechocystis* sp. strain PCC 6803 GAPDH2. It should be noted that this result is quite different from what has been reported for the chloroplastic GAPDH of higher plants, which is practically NADP specific, its affinity for the phosphorylated coenzyme being much higher (ca. 100-fold) (7, 16).

To our knowledge, an esterase activity has been described so far only for the classical glycolytic NAD-dependent GAPDH (44). We found that *Synechocystis* sp. strain PCC 6803 GAPDH2 also exhibited a high hydrolase activity (specific activities for purified natural and recombinant enzymes in the same range, 200 to 250 U/mg) when we used one of a variety of organic *p*-nitrophenylesters (either *p*-nitrophenylacetate, *p*-nitrophenylbutyrate or *p*-nitrophenylphosphate) as the substrate. The physiological significance of the esterase activity of cyanobacterial GAPDH2 remains to be investigated.

Western and Northern blot analysis of *gap-2* expression in *Synechocystis* sp. strain PCC 6803. Monospecific polyclonal antibodies raised in a rabbit against the electrophoretically pure natural *Synechocystis* sp. strain PCC 6803 GAPDH2 were used to analyze the expression of the *gap-2* gene at the protein level. These antibodies recognized in Western blots a single protein band of ca. 38 kDa, corresponding to the GAPDH2 subunit, in protein extracts either from this cyanobacterium or from *E. coli* W3CG bearing pFV8 (Fig. 4B; see also Fig. 1B). Immunotitration experiments showed that these antibodies were able to completely inhibit both NAD- and NADP-dependent GAPDH activities in total protein extracts from *Synechocystis* sp. strain PCC 6803 cells grown under either photoautrophic or heterotrophic conditions, indicating that the GAPDH2 should be the only enzyme with such activities present in these preparations (data not shown). Cross-reaction studies provided information on the possible structural and phylogenetic relationships of the *Synechocystis* sp. strain PCC 6803 GAPDH2 with homologous enzymes from diverse organisms (Fig. 4B). The antibodies were able to recognize not only the GAPDH2s of other cyanobacteria but also the chloroplastic NADP-dependent GAPDHs of plants and a variety of eukaryotic microalgae. A 38-kDa protein band was found in Western blots of cell extracts from all other cyanobacteria tested and from eukaryotic microalgae, whereas two clear bands, corresponding to *gapA* and *gapB* products, could be seen in higher plants. No bands were immunodetected in cell extracts containing only the glycolytic GAPDH, such as those from *E. coli* K-12 and DH5α (Fig. 1B) or from nonphotosynthetic protists, such as the ciliated protozoa *Tetrahymena pyriformis* and *Paramecium tetraurelia*, and diverse mammalian tissues (data not shown). The recombinant GAPDH1 (an NAD-specific cyanobacterial enzyme encoded by the *gapC*-like gene *gap-1*) of *Anabaena* sp. strain PCC 7120 (49) also was not recognized by these antibodies. It seems, therefore, that products of both the cyanobacterial *gap-2* and the *gapA* and *-B* genes of photosynthetic eukaryotes share structural characteristics that are not found in the products of the ubiquitous *gapC* superfamily (8, 21).

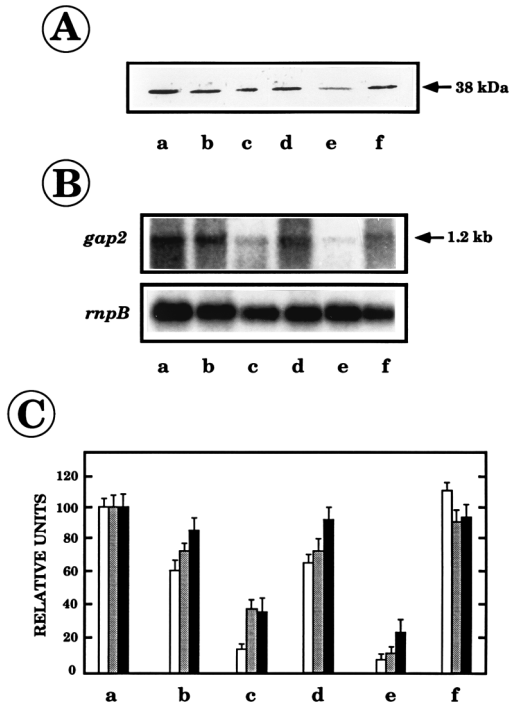

FIG. 5. Comparative analysis of GAPDH2 protein and *gap-2* transcript levels in *Synechocystis* sp. strain PCC 6803 cells grown under different trophic regimens. (A) Immunoblots of protein extracts from *Synechocystis* sp. strain PCC 6803, using the anti-GAPDH2 antibodies as for Fig. 4B. Lane a, nitrate/light; lane b, ammonium/light; lane c, nitrate plus glucose/light; lane d, N starvation/light; lane e, nitrate plus glucose/darkness; lane f, P starvation/light. Molecular size markers were electrophoresed in the same gel and stained for protein presence. About 70 μg of total protein was loaded per lane. (B) Northern blots of total RNA isolated from cells grown in the conditions described for panel A. Either a 0.94-kb internal *gap-2* fragment generated by PCR from plasmid pFV8 (top) or an internal fragment of *rnpB* which codes for *Synechocystis* sp. strain PCC 6803 RNase P RNA (loading control; bottom) was used as the probe. The arrow shows the position of the 1.2-kb *gap-2* transcript. RNA standards were electrophoresed in the same gel and stained with ethidium bromide. About 15 μg of total RNA was loaded per lane. (C) Correlation of the relative levels of enzymatic activity and the *gap-2* products, GAPDH2 protein, and *gap-2* mRNA transcript. These parameters were quantified in *Synechocystis* sp. strain PCC 6803 cell extracts from cells grown under the conditions specified for panel A. Data are means of three independent determinations and are expressed as percent relative units, the value for cells grown with nitrate being considered 100%. Open bars, specific activity (100%, 50 ± 3 mU/mg of protein); stippled bars, quantitation of the immunostained GAPDH2 protein; closed bars, quantitation of the 1.2-kb *gap-2* transcript RNA band after normalization with the loading control.

The highly specific antibodies for the cyanobacterial GAPDH2 allowed us to study the expression of *gap-2* at the protein level in *Synechocystis* sp. strain PCC 6803 without the possible interference of other GAPDHs that in principle might have been present in the crude preparations (Fig. 5A). On the other hand, to analyze the expression of *gap-2* at the mRNA

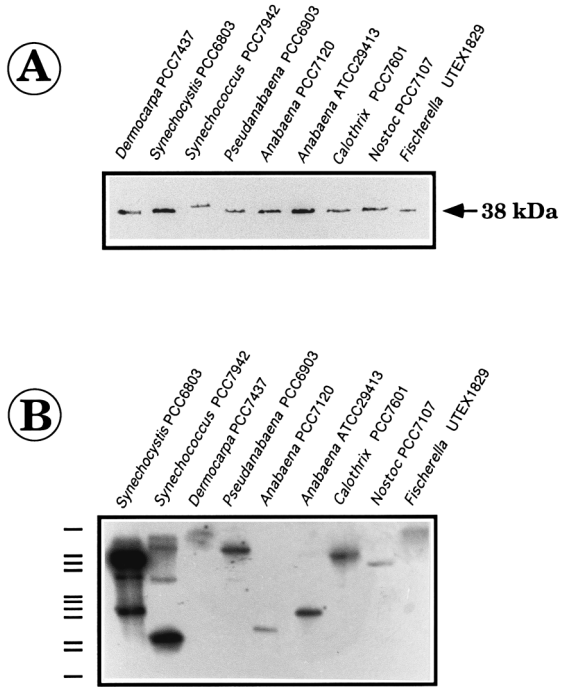

FIG. 6. Occurrence of both GAPDH2 and *gap-2* in strains representative of some cyanobacterial taxonomic groups. (A) Immunodetection of the GAPDH2 protein in cell extracts of different cyanobacteria by Western blotting using the anti-GAPDH2 antibodies raised against the *Synechocystis* sp. strain PCC 6803 enzyme. About 70 μg of total protein was loaded per lane. (B) Southern blot analysis of genomic DNAs from diverse cyanobacteria digested with *Hin*dIII and probed with a ^{32}P-radiolabelled 0.94-kb internal *gap-2* fragment generated by PCR from pFV8. The strains are identified by their collection numbers. The positions of *Eco*RI-*Hin*dIII-restricted λ DNA fragments used as standards (in the range of 21 to 0.5 kb) are indicated on the left side.

level, a 0.94-kb internal fragment of *Synechocystis* sp. strain PCC 6803 *gap-2* was used as a probe in Northern blot analysis of total RNA samples isolated from cultures of this cyanobacterium; a single transcript of about 1.2 kb, the expected size for the *gap-2* product, was found in all cases (Fig. 5B). The RNA component of the ribozyme RNase P, a housekeeping enzyme in *Synechocystis* sp. strain PCC 6803 (50), was used as a loading control. Both Western and Northern blot analyses clearly indicated that the expression of *Synechocystis* sp. strain PCC 6803 *gap-2* was affected by the trophic conditions of the cell cultures. A good correlation was found among specific activity values and immunodetected GAPDH2 protein and *gap-2* transcript levels in the different culture conditions tested (Fig. 5C). The main effect was the marked decrease in all parameters up to ca. 10% of the control values (photoautrophic cells) by the addition of glucose, a sugar metabolizable by this cyanobacterium (1, 15), either in the light (photoheterotrophy) or in darkness (chemoheterotrophy). It should be noted that cells cultured in the light with glucose and 10 μM DCMU (strict photoheterotrophy) presented levels similar to those cultured in heterotrophic conditions (data not shown). In contrast, the rather high levels observed in photoautotrophically grown cells were slightly affected either by the source of inorganic nitrogen (ca. 30% decrease when grown with ammonia instead of nitrate) or by nutritional stress conditions such as N starvation or P limitation, but in the last two conditions a decrease of total cellular protein was also found. An apparent activation of the enzyme was observed only in P-depleted cells. In summary, Northern and Western blots indicate that *gap-2* is sufficiently expressed and GAPDH2 appears in detectable levels in *Synechocystis* sp. strain PCC 6803 cells growing under different trophic conditions, even in strict chemoheterotrophy when no photosynthesis is performed at all, so that it seems able to accomplish by itself all potential metabolic functions, without the need for another *gap* product.

Our data on GAPDH2 regulation shed new light on several contradictory reports in the early literature concerning specific activity measurements in cell extracts of cyanobacteria (3, 13, 24, 32, 33). The good correlation found between specific activity, GAPDH2 protein, and mRNA *gap-2* transcript levels during the dramatic decrease of these parameters observed after glucose addition to *Synechocystis* sp. strain PCC 6803 cultures indicates that no process of enzyme activation or inactivation is involved in these changes. Thus, regulation by the presence of the metabolizable sugar seems to be due to changes in the mRNA levels rather than to GAPDH2 activity modulation. Glucose should not be regarded as a proper nutrient but rather as a toxic compound since its addition to photoautotrophic cultures of *Synechocystis* sp. strain PCC 6803 promotes at first a high cell mortality, and cultures adapted to photoheterotrophic conditions exhibit lower growth rates than photoautotrophic cells (1, 15).

The results for *Synechocystis* sp. strain PCC 6803 are in sharp contrast with our previous observations on the eukaryotic microalga *Chlorella fusca* (40). In the latter organism, the presence of the sugar caused a marked decrease of the NADP-

TABLE 2. NAD(P)-dependent GAPDH specific activities of cell extracts from several representative strains of some cyanobacterial taxonomic groups[a]

Cyanobacterial strain	Sp act (mU/mg of protein)[b]			NAD/NADP activity ratio
	NAD dependent	NADP dependent	NAD plus NADP in assay	
Chroococcales				
Synechocystis sp. strain PCC 6803	50	49	45	1.02
Synechococcus sp. strain PCC 7942	14	24	37	0.58
Pleurocapsales				
Dermocarpa sp. strain PCC 7437	63	72	69	0.89
Oscillatoriales				
Pseudanabaena sp. strain PCC 6903	26	27	22	0.96
Nostocales				
Anabaena sp. strain PCC 7120	25	21	23	1.19
Anabaena sp. strain ATCC 29413	71	60	58	1.18
Nostoc sp. strain PCC 7107	38	31	34	1.22
Calothrix sp. strain PCC 7601	13	11	10	1.18
Stigonematales				
Fischerella sp. strain UTEX 1829	22	21	19	1.05

[a] All cyanobacteria were grown photoautotrophically in BG11 medium (nitrate as the nitrogen source) under continuous white light (25 W/m^2) and bubbling with air plus 1.5% (vol/vol) CO_2.

[b] Data are the means of at least three independent determinations using late-exponential-phase cultures; the standard error was less than 15% in all cases.

dependent GAPDH activity and protein amount to undetectable values, but photoheterotrophic cultures exhibited even higher growth rates than the photoautotrophic ones. Like other photosynthetic eukaryotes, *C. fusca* has a cytosolic NAD-dependent GAPDH, different from the chloroplastic NADP-dependent enzyme, that participates in the catabolic pathways involved in sugar assimilation (29, 40). The different behavior of *Synechocystis* sp. strain PCC 6803 might be due to the capacity of glucose to act in this organism as a catabolic repressor of the GAPDH2 involved in photosynthetic carbon assimilation. In other words, if no GAPDH other than GAPDH2 could efficiently accomplish carbohydrate catabolism in *Synechocystis* sp. strain PCC 6803, repression of *gap-2* expression would necessarily lead to an impairment of growth under photoheterotrophic conditions, as was found to be the case (15). Overall, our results are in agreement with the amphibolic role assigned to GAPDH2 and suggest that only this enzyme is functional in this cyanobacterium under both photosynthetic and heterotrophic growth conditions.

Since the *Synechocystis* sp. strain PCC 6803 GAPDH2 is detected at conspicuous levels under both phototrophic and heterotrophic conditions, this enzyme could efficiently function either in the photosynthetic RPP pathway or in carbohydrate catabolism. Kinetic data indicate, however, that the *Synechocystis* sp. strain PCC 6803 GAPDH2 exhibits in the reductive (anabolic) reaction a twofold-higher catalytic efficiency (V_{max}/K_m) with NADH than with NADPH, despite equal K_m values for both pyridine nucleotides. In contrast, typical chloroplastic enzymes are about 100 times more efficient with NADPH (7). Thus, GAPDH2 seems in this respect to be somewhat related to GAPDH of photosynthetic bacteria, which perform anoxygenic photosynthesis using NADH as the reducing cofactor in the RPP pathway (9). Levels of NAD(P)H in cyanobacterial cells might change significantly depending on the environmental trophic conditions, being much higher in light-growing cells, providing enough substrate for the anabolic reactions, and low in darkness due to cyanobacterial respiration (38, 43). It has been postulated that the intracellular NAD(P)H/NAD(P) ratio could regulate the cyanobacterial glucose-6-phosphate and 6-phosphogluconate dehydrogenases (43, 45, 46). The possible response of *Synechocystis* sp. strain PCC 6803 GAPDH2, which unlike other photosynthetic GAPDHs seems to prefer NADH to NADPH in the reductive reaction, to changes of these metabolic parameters may have a regulatory significance worth investigating.

Occurrence of *gap-2* and GAPDH2 among cyanobacteria. Genomic DNA and total protein preparations obtained from photoautotrophically grown cells of several strains representative of some cyanobacterial taxonomic groups were tested for both the *gap-2* gene and the GAPDH2 protein. The following strains were used for this purpose: *Synechocystis* sp. strain PCC 6803, *Synechococcus* sp. strain PCC 7942, *Dermocarpa* sp. strain PCC 7437, *Pseudanabaena* sp. strain PCC 6903, *Anabaena* sp. strains PCC 7120 and ATCC 29413, *Nostoc* sp. strain PCC 7107, *Calothrix* sp. strain PCC 7601, and *Fischerella* sp. strain UTEX 1829. Western blot analysis of the extracts using the anti-GAPDH2 antibodies clearly revealed that the product of the *gap-2* gene was present in all of them (Fig. 6A), thus proving the general expression of this gene among cyanobacteria. A single immunostained protein band of 38 kDa was seen in the protein extracts from these strains, except in the case of *Synechococcus* sp. strain PCC 7942, which showed a slightly larger band. This finding is in agreement with a recent report showing that this cyanobacterium presents a peculiar GAPDH2 with an additional amino acid sequence at the carboxy terminus (53). On the other hand, when either a 2.1-kb-long *Ase*I fragment of the pFV8 insert containing the *Synechocystis* sp. strain PCC 6803 *gap-2* gene (not shown) or a 0.94-kb PCR-generated internal fragment of the gene was used as a probe in Southern blot experiments with *Hin*dIII-restricted cyanobacterial DNA, clear bands could be detected in all cases (Fig. 6B), indicating again a wide distribution of this gene among the strains tested.

Levels of both NAD- and NADP-dependent GAPDH activities were also determined in the total protein extracts used in the experiment described above. As shown in Table 2, very similar levels were found for both activities in the different cyanobacterial extracts, values of around 1 being observed for the NAD-/NADP-dependent activity ratio, again with the remarkable exception of *Synechococcus* sp. strain PCC 7942, in which this ratio was clearly lower. This latter result could be expected since the GAPDH2 from this cyanobacterium exhibits lower affinity for NAD (53), another aspect in which this enzyme differs from those of *Synechocystis* sp. strain PCC 6803 and *A. variabilis*. No additivity between both activities (that is, no significant increase of enzymatic activity in the presence of both coenzymes) was observed, strongly suggesting that both pyridine nucleotides compete for the same active site and that only one enzyme is present in the extracts. It is noteworthy that, once more, this was not the case for the peculiar GAPDH2 of *Synechococcus* sp. strain PCC 7942. Whether this is due either to a different kind of GAPDH2 regulation or to the presence of another NAD-dependent GAPDH remains to be established. However, although *Synechococcus* sp. strain PCC 7942 has two different *gapC*-like genes, *gap-1* and *gap-3*, its peculiar GAPDH2 is the only GAPDH enzyme present in crude extracts, as shown by recent biochemical studies (48). It is also interesting that in the photosynthetic protist *Cyanophora paradoxa*, a single NAD(P)-dependent GAPDH, also exhibiting a clear dual dependence for the nucleotide cofactor, was characterized in the cyanelle, whereas the NAD-dependent glycolytic enzyme was found only in the cytosol (39). Since this cyanobacterium-like organelle has been postulated as an evolutionary relic reminiscent of the ancient cyanobacterial endosymbiont precursor of photosynthetic plastids, these findings agree with our proposal on the amphibolic character of the cyanobacterial GAPDH2.

ACKNOWLEDGMENTS

We thank A. Vioque for kindly providing the *Synechocystis* sp. strain PCC 6803 genomic library and B. Bachman and A. Plücktung for the *E. coli gap* mutants DF221 and W3CG, respectively. We owe many thanks to E. Flores and J. R. Pérez-Castiñeira for comments on the manuscript and W. Löffelhardt for the amino-terminal sequencing of the GAPDH2 proteins at the Institut für Biochemie und Molekulare Zellbiologie, University of Vienna (Austria).

This work was supported by DGICYT grants PB-91/0085 and PB-94/0033 from the Spanish government and grant PAI CVI-0198 from the Andalusian government.

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