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NADP$^+$-Isocitrate Dehydrogenase from the Cyanobacterium *Anabaena* sp. Strain PCC 7120: Purification and Characterization of the Enzyme and Cloning, Sequencing, and Disruption of the *icd* Gene

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NADP$^+$-isocitrate dehydrogenase (NADP$^+$-IDH) from the dinitrogen-fixing filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 was purified to homogeneity. The native enzyme is composed of two identical subunits (M_r, 57,000) and cross-reacts with antibodies obtained against the previously purified NADP$^+$-IDH from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *Anabaena* NADP$^+$-IDH resembles in its physicochemical and kinetic parameters the typical dimeric IDHs from prokaryotes. The gene encoding *Anabaena* NADP$^+$-IDH was cloned by complementation of an *Escherichia coli icd* mutant with an *Anabaena* genomic library. The complementing DNA was located on a 6-kb fragment. It encodes an NADP$^+$-IDH that has the same mobility as that of *Anabaena* NADP$^+$-IDH on nondenaturing polyacrylamide gels. The *icd* gene was subcloned and sequenced. Translation of the nucleotide sequence gave a polypeptide of 473 amino acids that showed high sequence similarity to the *E. coli* enzyme (59% identity) and with IDH1 and IDH2, the two subunits of the heteromultimeric NAD$^+$-IDH from *Saccharomyces cerevisiae* (30 to 35% identity); however, a low level of similarity to NADP$^+$-IDHs of eukaryotic origin was found (23% identity). Furthermore, *Anabaena* NADP$^+$-IDH contains a 44-residue amino acid sequence in its central region that is absent in the other IDHs so far sequenced. Attempts to generate *icd* mutants by insertional mutagenesis were unsuccessful, suggesting an essential role of IDH in *Anabaena* sp. strain PCC 7120.

Cyanobacteria are oxygenic photosynthetic prokaryotes that have an incomplete tricarboxylic acid cycle because they lack α-ketoglutarate dehydrogenase and succinyl-coenzyme A synthetase activities (42, 49). Thus, the isocitrate dehydrogenase (IDH) reaction constitutes a terminal step in carbon flow in these organisms. The product, α-ketoglutarate, provides the carbon skeleton required for ammonium assimilation through the glutamine synthetase-glutamate synthase pathway (35) and represents a key metabolite in the linking of nitrogen and carbon metabolism. On the other hand, the NADPH formed by the IDH reaction has been proposed to play an essential role in nitrogen-fixing cyanobacteria by reducing ferredoxin, which is the electron carrier in the N_2 fixation of heterocysts (5).

Although NADP$^+$-dependent and NAD$^+$-dependent IDHs (EC 1.1.1.42 and EC 1.1.1.41) have been described in prokaryotes, most bacteria have only the NADP$^+$-linked enzyme (10). In certain cases, the same enzyme is capable of using both pyridine nucleotides (31). The most extensively studied prokaryotic NADP$^+$-IDH is that of *Escherichia coli*, which is a homodimer regulated by phosphorylation (30) and is involved in the Krebs cycle (50).

In eukaryotes, oxidative decarboxylation of isocitrate is catalyzed by three different isozymes that vary in subunit structure and cofactor specificity. NAD$^+$-dependent IDH from eukaryotic sources functions as an oligomeric enzyme that is subject to extensive allosteric regulation and is implicated in energy production in mitochondria. There are also cytosolic, mitochondrial, and chloroplastic forms of NADP$^+$-specific IDH whose metabolic functions are unclear but which presumably provide precursors for various biosynthetic pathways (10).

In cyanobacteria, IDH is strictly dependent on NADP$^+$ and no NAD$^+$-IDH activity has been reported (18, 38, 40). We have previously purified and characterized the NADP$^+$-IDH from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (38). The enzyme is composed of two identical subunits (M_r, 57,000) and shows kinetic and physicochemical parameters similar to those of the NADP$^+$-IDH from *E. coli* (38, 43).

Genes encoding prokaryotic NADP$^+$-IDH have been cloned from *E. coli* (50) and *Thermus thermophilus* (36) and sequenced. NAD$^+$- and NADP$^+$-IDH genes have also been cloned from eukaryotic sources and sequenced (11, 12, 19, 20, 23, 48, 51). Analysis of the deduced amino acid sequences indicates that the primary structure of the prokaryotic NADP$^+$-IDH is more related to that of eukaryotic NAD$^+$-IDH than to that of eukaryotic NADP$^+$-IDH (12). However, all of the IDHs contain conserved amino acid residues and a specific sequence motif related to isopropylmalate dehydrogenase (IMDH) (48).

In this report, we describe the cloning by complementation of an *E. coli icd* mutant of the gene encoding the NADP$^+$-IDH from the N_2-fixing filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. The predicted amino acid sequence of the *Anabaena* NADP$^+$-IDH is similar to that of the *E. coli* enzyme but contains an insertion which seems to be specific for the cyanobacterial sequence. We have also purified and character-

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ized the NADP+-IDH from this cyanobacterium and compared its molecular properties with those of other prokaryotic IDHs. On the other hand, our results indicate that the *icd* gene is essential for *Anabaena* growth.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. This study was carried out with the cyanobacterium *Anabaena* sp. strain PCC 7120 cultivated photoautotrophically at 30°C with shaking in BG11 medium (44). For purification, it was grown in 20-liter Pyrex bottles under N_2-fixing conditions and bubbled with air. Cells were harvested by continuous-flow centrifugation at 7,000 × *g* and kept frozen at −20°C until needed. Selective growth of exconjugants was in BG11 medium supplemented with neomycin (25 or 100 μg ml^{-1}). The growth phenotype of strain MA1 was tested in 50-ml cultures of BG11 medium, $BG11_0$ (without combined nitrogen), or modified BG11 medium with 5 mM NH_4Cl instead of nitrate; the media were buffered with 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and, where indicated, supplemented with 5 mM α-ketoglutarate, 3 mM L-proline, or 0.5 mM glutamate. All of the liquid cultures of MA1 contained 10 μg of neomycin ml^{-1}.

E. coli DH5α (Bethesda Research Laboratories), used for all plasmid constructions, and *E. coli* HB101 (6), used for conjugation, were grown in Luria broth as described by Sambrook et al. (46). *E. coli* DEK 2004, a *trp icd recA* mutant strain, was grown in Luria broth supplemented with 100 μg of ampicillin ml^{-1} when necessary or in morpholinepropanesulfonic acid (MOPS)-based medium (39) supplemented with glucose (0.5%) and the appropriate amino acids (0.5 mM).

The plasmids used were pMA1, pMA2, and pMA3, which are pBluescript SK(+) derivatives with fragments of 6, 3, and 2.1 kb, respectively, of *Anabaena* genomic DNA that contain the *icd* gene. pRL277 is a streptomycin-spectinomycin-resistant mobilizable vector (4), pRL443 is a conjugative plasmid (15), pRL528 is a helper plasmid for conjugation (15), and pTK513 is a pUC-derivative plasmid containing the *icd* gene from *E. coli*.

Purification and characterization of NADP-specific IDH. NADP+-specific IDH activity was measured in 50 mM potassium phosphate (pH 7.5) containing 3 mM $MgCl_2$, 1 mM isocitrate, and 0.4 mM NADP+ in a final volume of 1 ml, following the reduction of NADP+ at 340 nm (38). Units are expressed as micromoles of NADPH produced per minute. Protein concentrations were determined by the method of Bradford (7).

For enzyme purification, frozen cells (60 g) were thawed in 150 ml of 30 mM Tris-HCl (pH 7.5) containing 1 mM sodium citrate, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 5% glycerol (buffer A); phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. The mixture was sonically disrupted (20 kHz, 75 W) for 5 min (in 30-s periods) in a Branson 250 Sonifier.

The homogenate was cleared by centrifugation at 18,000 × *g* for 20 min. The supernatant (130 ml) was loaded onto a DEAE-cellulose column (3.5 by 15 cm), and after washing with buffer A, elution of the enzyme was performed with a linear NaCl gradient (0 to 0.4 M) in 400 ml of the same buffer. Fractions containing the single peak of activity (about 0.1 M NaCl) were pooled and passed through a Reactive Red 120-agarose column (2.5 by 7 cm). This column was washed with buffer A, and elution of IDH was carried out by a linear gradient (0 to 0.4 M NaCl) in 140 ml of the same buffer. The fractions with high levels of IDH activity (about 0.35 M NaCl) were diluted fourfold, combined, and loaded onto a second Reactive Red 120-agarose column (1 by 10 cm). IDH activity was eluted with a gradient of NADP+ (0 to 2 mM) prepared in 30 ml of buffer A supplemented with 50 mM NaCl. IDH eluted at about 1.8 mM NADP+, and all of the fractions that showed activity were pooled, equilibrated up to 1 M ammonium sulfate, and applied to a Phenyl-Sepharose column (1 by 10 cm) previously equilibrated with buffer A containing 1 M ammonium sulfate. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient (1 to 0 M) of ammonium sulfate in buffer A. Active fractions were concentrated, and the purity of NADP+-IDH was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

The native molecular mass of NADP+-IDH from *Anabaena* sp. was calculated by gel filtration chromatography on a Sephacryl S-300 column (1.6 by 32 cm) equilibrated with 30 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. Standard proteins for column calibration were thyroglobulin (660 kDa), aldolase (158 kDa), ovalbumin (45 kDa), and chymotrypsinogen (25 kDa).

The isoelectric point of *Anabaena* NADP+-IDH was determined by rapid isoelectric focusing in a vertical polyacrylamide minigel system as described by Robertson et al. (45), utilizing a linear pH gradient (2.50 to 6.50). The pH effect was studied at 30°C by using 50 mM potassium phosphate (pH 6.5 to 7.5) and 50 mM Tris-HCl (pH 7.5 to 9.5).

Kinetic analysis of NADP+-IDH was carried out at 30°C and pH 7.5. K_m values for isocitrate, NADP+, Mg^{2+}, and Mn^{2+} were determined by the single-variable method. Each K_m value was obtained at saturating concentrations of all of the other substrates. Reaction mixtures contained 50 mM potassium phosphate (for assays with Mg^{2+}) or 50 mM triethanolamine hydrochloride (for assays with Mn^{2+}) and different concentrations of substrates and cofactor. The reaction was started by addition of the enzyme.

PAGE. Enzyme purification was monitored by SDS-PAGE as described by Laemmli (28), with 12% (wt/vol) acrylamide slab gels. Nondenaturing gels contained 10% (wt/vol) acrylamide in the resolving phase and 4.5% (wt/vol) acrylamide in the stacking phase. Protein bands were stained with 0.25% Coomassie brilliant blue R-250. Marker proteins were SDS-PAGE molecular mass standards (low range) from Bio-Rad Laboratories. Identification of NADP+-IDH activity by nondenaturing gel electrophoresis was carried out by NADPH fluorescence dependent on isocitrate. Gels were removed from the glass plates and submerged in a staining solution containing 200 mM Tris (pH 7.9), 0.4 mM NADP+, 15 mM $MgCl_2$, 8 mM isocitrate, 0.4 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide, and 0.5 mM phenazine methosulfate.

Immunoprecipitation. Samples of purified NADP+-IDH from *Anabaena* and *Synechocystis* cyanobacteria were incubated with increasing amounts of antibody raised against *Synechocystis* NADP+-IDH (38) for 12 h at 4°C. Insoluble antigen-immunoglobulin G complexes were pelleted by centrifugation for 15 min at 15,000 × *g*, and the enzyme activity in the supernatant fraction was measured. Immunoprecipitation of cloned NADP+-IDH from *Anabaena* and *E. coli* bacteria was carried out with crude extracts of *E. coli icd* mutant DEK 2004 transformed with pMA3 and pTK513, respectively, by the procedure described above.

Complementation of the *E. coli icd* mutant. Complementation of glutamate auxotroph *E. coli* DEK 2004 with a gene library from wild-type *Anabaena* sp. strain PCC 7120 (32) was performed as follows. Competent cells of *E. coli* DEK 2004 were transformed with the gene bank by the standard proce-

dure (46). After 1 h of enrichment at 37°C in Luria broth, the cells were washed with MOPS medium and plated on solid MOPS medium supplemented with glucose (0.5%), tryptophan (0.5 mM), and ampicillin (40 μg ml^{-1}). Plates were incubated at 37°C until glutamate prototroph colonies appeared.

Recombinant DNA techniques and nucleotide sequencing. Total DNA from cyanobacteria was isolated as described by Cai and Wolk (8). Plasmid isolation from *E. coli*, transformation of *E. coli*, restriction, and ligation with T4 ligase were performed by standard procedures (1, 46). DNA fragments were purified from agarose gels with the Gene-Clean Kit (Bio 101, Inc). For Southern hybridizations, DNA was digested and fragments were electrophoresed in 0.7% agarose gels in a Tris-borate-EDTA buffer system (46). Transfer of DNA to Z-Probe membranes (Bio-Rad Laboratories) was done under vacuum, and Southern blot hybridizations were performed as described in reference 1. DNA probes were ^{32}P labelled by the random primer technique with [α-^{32}P]dCTP.

Sequencing of the DNA fragment containing the *icd* gene was carried out by the dideoxy-chain termination method (47) with Sequenase version 2.0 (U.S. Biochemical Corp.). Nested unidirectional deletions were generated with the double-stranded Nested Deletion Kit from Pharmacia LKB. Computer searching for homologies was done with the FASTA program, and alignments were produced with the Pileup program using default parameters (13).

Insertional mutagenesis of the *icd* gene in *Anabaena* sp. strain PCC 7120. The method of *sacB*-mediated positive selection for double recombinants in *Anabaena* sp. strain PCC 7120 (8) was used to mutagenize the *icd* gene. An Nm^r cassette (C.K1) (14), *Hin*cII ended, was cloned into an *Xmn*I site of pMA2 internal to the *icd* gene. The resulting plasmid was *Hin*cII-*Sma*I digested, and the fragment containing the disrupted *icd* gene was cloned into the *Xba*I site of *sacB* mobilizable vector pRL277. This plasmid was transferred to *Anabaena* sp. strain PCC 7120 by conjugation with conjugal plasmid pRL443 and helper plasmid pRL528 as described by Elhai and Wolk (15). After triparental mating, cells were spread onto filters (REC-85; Nuclepore), set atop solidified BG11 medium supplemented with 5% Luria broth, and incubated for 45 h at 30°C under low light. Filters were then transferred to BG11 plates containing 25 μg of neomycin ml^{-1} and further incubated under growth conditions for 25 days; exconjugants were restreaked on agar plates containing neomycin and 5% (wt/vol) sucrose. Double recombinants were identified by their sucrose-resistant, neomycin-resistant, streptomycin-spectinomycin-sensitive phenotype (streptomycin-spectinomycin resistance was provided by the vector part of the transferred plasmid) and by Southern blot analysis.

Nucleotide sequence accession number. The EMBL-GenBank accession number for the sequence described here is X77654.

RESULTS

Purification and properties of $NADP^+$-IDH. We previously determined that $NADP^+$-IDH activity in *Anabaena* sp. strain PCC 7120 grown on different nitrogen sources exhibits the highest values under dinitrogen-fixing conditions with respect to cells grown in a combined nitrogen source (38), so purification was done with cells grown in nitrogen-free medium ($BG11_0$).

$NADP^+$-IDH from *Anabaena* sp. strain PCC 7120 was very labile in response to column chromatographic techniques; however, reasonable recoveries and maintenance of enzyme activity were achieved by adding stabilizing agents (2 mM 2-mercaptoethanol, 1 mM sodium citrate, 5 mM $MgCl_2$, and 5% [vol/vol] glycerol) to the separation buffer. In Materials and Methods, a purification scheme is described that utilizes sequential column chromatography steps, including DEAE-cellulose, Reactive Red-agarose and Phenyl-Sepharose.

TABLE 1. Purification of $NADP^+$-IDH[a] from *Anabaena* sp. strain PCC 7120

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	7,450	433	0.058	100	1
DEAE-cellulose	884	260	0.29	60	5
First Reactive Red 120-agarose	47.36	131	2.76	30	48
Second Reactive Red 120-agarose	6.77	155	22.94	36	393
Phenyl-Sepharose	4.33	108	24.94	25	430

[a] The enzyme was purified from 60 g (fresh mass) of *Anabaena* sp. strain PCC 7120.

As shown in Table 1, this procedure gave 430-fold purification and yielded 4.33 mg of the purified enzyme from 60 g of cells. Figure 1 shows an SDS-polyacrylamide gel of samples from each step of the purification scheme; the final product appeared to be a single, essentially homogeneous polypeptide with a molecular weight of approximately 57,000. The enzyme has a native molecular mass of 108 kDa, as determined by molecular-exclusion chromatography on Sephacryl S-300 (data not shown). These data suggest that the *Anabaena* enzyme is a homodimer, as are other $NADP^+$-specific IDHs from prokaryotes and eukaryotes. Isoelectric focusing of the purified $NADP^+$-IDH gave an isoelectric point of 5.5. The heat inactivation profile indicated that 50% of the enzyme activity remained after 20 min of incubation at 60°C. $NADP^+$-IDH was also found to be catalytically active over a wide pH range, with 75% of maximal activity occurring between pHs 7.5 and 9.5 and optimal activity at pH 8.5.

The enzyme exhibited an absolute requirement for divalent cations. Various metal ions (500 μM) were tested in the standard reaction mixture. $NADP^+$-IDH activity was maximal with Mn^{2+}; activities obtained with other cations as activators

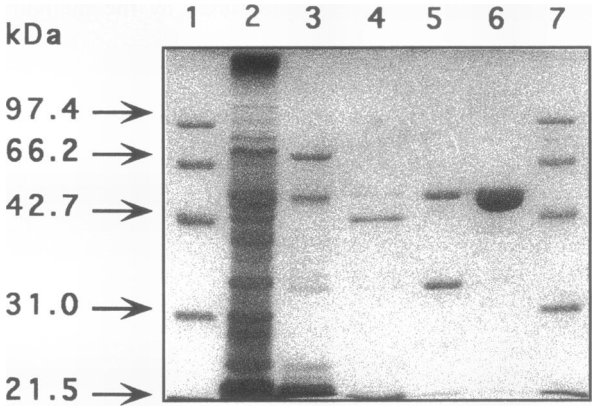

FIG. 1. SDS-PAGE analysis of $NADP^+$-IDH purification. Lanes: 1 and 7, marker proteins; 2, crude extract; 3, DEAE-cellulose eluate; 4 and 5, first and second Reactive Red 120-agarose eluates, respectively; 6, purified IDH (17 μg).

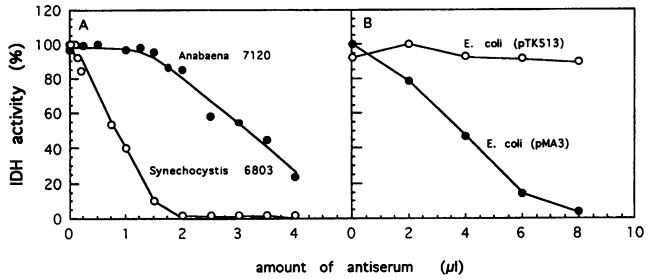

FIG. 2. Immunotitration curves of NADP+-IDH activity. (A) Purified NADP+-IDHs (0.04 U [each]) from *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803 were incubated with increasing volumes of antiserum. (B) NADP+-IDHs from crude extracts of *E. coli* DEK 2004(pTK513) containing the *E. coli icd* gene and *E. coli* DEK 2004(pMA3) containing the *Anabaena icd* gene were immunoprecipitated with increasing amounts of antiserum. The enzyme activity remaining in supernatants was measured as described in Materials and Methods.

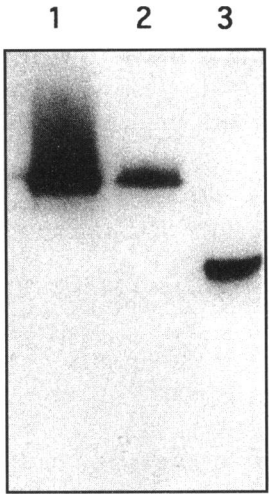

FIG. 3. IDH activity staining of native PAGE of purified IDH and extracts of complemented *E. coli* DEK 2004. Lanes: 1, purified *Anabaena* IDH; 2, crude extract from *E. coli* DEK 2004(pMA3) containing the *Anabaena icd* gene; 3, crude extract from *E. coli* DEK 2004(pTK513) containing the *E. coli icd* gene. See Materials and Methods for details.

of the enzyme were as follows (relative to activity with Mn^{2+}): Mg^{2+}, 41%; Co^{2+}, 46%; and Ni^{2+}, 10%.

Apparent K_m values were 4.2 and 9.3 μM for DL-isocitrate and NADP+, respectively, with Mn^{2+} as a divalent cation and 61.2 and 13.6 μM for DL-isocitrate and NADP+, respectively, with Mg^{2+} as a cofactor.

To identify metabolites that could modulate NADP+-IDH activity, we tested Krebs cycle metabolites and amino acids directly related to the glutamine synthetase-glutamate synthase pathway, such as glutamate, glutamine, and proline. Under all of the conditions tested, no significative inhibition of the enzyme was found with a 10 mM concentration of any of these metabolites. Adenine nucleotides AMP, ADP, and ATP inhibited NADP+-IDH activity by 23, 50, and 70%, respectively, when present at 20 mM. The reduced pyridine nucleotide NADPH inhibited NADP+-IDH activity by 28% when it was added to the assay mixture at 0.25 mM. The presence of either oxaloacetate or glyoxylate independently at 10 mM led to 30 and 8% inhibition, respectively, but the combination of 1 mM oxaloacetate plus 1 mM glyoxylate inhibited the activity by 99%, probably by mimicking the substrate, isocitrate.

Anabaena NADP+-IDH cross-reacted with antibodies raised against the purified NADP+-IDH from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (38), as shown in the immunotitration curve in Fig. 2A.

Isolation of the *icd* gene. The fact that the molecular and structural properties of *Anabaena* NADP+-IDH resemble those of the *E. coli* enzyme, taken together with the existence of *icd* mutants of *E. coli*, led us to attempt isolation of the *Anabaena icd* gene by complementation of an *E. coli icd* mutant strain.

E. coli DEK 2004, an *icd* mutant and glutamate auxotroph, was transformed with an *Anabaena* genomic library as described in Materials and Methods. After selection on minimal medium without added glutamate, a single complementing plasmid was isolated from 1 of more than 5,000 independent transformants and was found to contain a 6-kb *Anabaena* genomic DNA fragment. Complementation was confirmed by transforming DEK 2004 with this plasmid (designated pMA1), and the presence of NADP+-IDH was verified by enzyme assay using extracts from strain DEK 2004(pMA1). The gene complementing the IDH defect was localized to a 2.1-kb *Bst*XI-*Hin*dIII fragment.

Activity staining in native gel electrophoresis showed that the relative mobility of the NADP+-IDH activity encoded by the cloned DNA was identical to that of the NADP+-IDH purified from *Anabaena* extracts but different from that of *E. coli* IDH (Fig. 3). Furthermore, the polyclonal antibodies raised against purified *Synechocystis* NADP+-IDH, which cross-reacted with purified *Anabaena* NADP+-IDH (Fig. 2A), were used in immunoprecipitation tests to determine the immunological properties of the cloned protein relative to NADP+-IDH in crude cell extracts of wild-type *Anabaena* sp. strain PCC 7120, as well as authentic *E. coli* IDH. The anti-NADP+-IDH serum immunoprecipitated the IDH activity present in DEK 2004(pMA3) extracts but not that present in DEK 2004(pTK513) extracts (Fig. 2B), confirming that the NADP+-IDH expressed from pMA3 was the *Anabaena* NADP+-IDH.

Nucleotide sequence analysis. Nested deletions were generated from pMA3 with exonuclease III, and a number of the resulting subclones were checked for the ability to complement the *E. coli icd* mutant and used for sequencing. Both strands of DNA were sequenced. The complete nucleotide sequence of the *icd* gene and translation of the open reading frame into a 473-residue amino acid sequence are shown in Fig. 4. The coding region ends with a TAA stop codon and can encode a polypeptide with a calculated molecular mass of 52,194 Da, which is similar to the molecular mass determined for the purified *Anabaena* NADP+-IDH subunit.

A putative ribosome-binding site (GGAG) is present 10 nucleotides upstream from the putative translation start site (Fig. 5). In the region of DNA upstream of the ATG codon, sequences related to the −35 and −10 consensus promoter regions of *Anabaena* genes were not identified, but six imperfect repeats of a seven-nucleotide sequence (consensus, 5′-CCCCAAT-3′) was found.

A search of the SWISS-PROT and EMBL-GenBank data bases revealed that the *Anabaena* NADP+-IDH is homologous to other IDHs and IMDHs sequenced from prokaryotic or eukaryotic organisms (Fig. 5) and contains the IDH and IMDH consensus sequence (amino acids 358 to 377). It is worth noting that IMDH from another cyanobacterium, *Spir-*

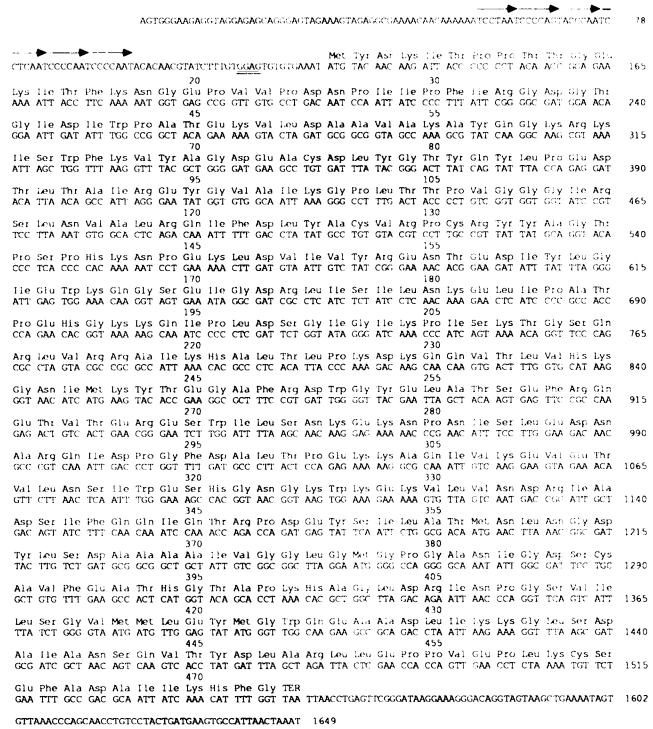

FIG. 4. Nucleotide sequence of the *Anabaena* sp. strain PCC 7120 *icd* gene and deduced amino acid sequence of NADP$^+$-IDH. A 1,649-bp nucleotide sequence containing the *Anabaena icd* gene was determined as described in Materials and Methods. The sequence was translated starting with the first methionine codon in the open reading frame as shown. A putative ribosome-binding site is underlined. Arrows denote six imperfect direct repeats of a heptanucleotide in the 5′ upstream region of the *icd* gene.

ulina platensis (3), has 30% sequence identity with respect to *Anabaena* NADP$^+$-IDH (data not shown). Multiple alignment of several IDH sequences (Fig. 5) indicated that *Anabaena* NADP$^+$-IDH is more similar to the *E. coli* IDH (59% identity) than to the *T. thermophilus* IDH (42% identity), the only other prokaryotic IDH which has been sequenced. On the other hand, with respect to eukaryotic IDHs, the similarity to NAD$^+$-IDH is greater than that to NADP$^+$-IDHs, as can be easily observed in the case of yeast sequences.

Disruption of the *icd* gene. To establish the role of the NADP$^+$-IDH in *Anabaena* sp. strain PCC 7120, an effort was made to isolate an *icd* mutant. An inactivated version of the cloned gene was constructed by insertion of an Nm^r cassette (C.K1) into pMA2. Figure 6A shows the structure of the *icd* genomic region in wild-type *Anabaena* sp. strain PCC 7120 and the insertion of the *npt* gene cassette. The inactivated gene (*icd*:CK1) was cloned into mobilizable vector pRL277, which contains the *sacB* gene from *Bacillus subtilis* and confers sensitivity to sucrose, allowing positive selection for double recombinants in *Anabaena* sp. strain PCC 7120 (8). After transfer of the plasmid by conjugation from *E. coli* to *Anabaena* sp. strain PCC 7120, selection of double recombinants as Nm^r Suc^r clones was carried out (see Materials and Methods for details).

Only one colony selected as Nm^r Suc^r was found to be Sr^s Sp^s, a phenotype suggestive of double recombination. Total DNAs from this strain, MA1 (*icd*:CK1), and from wild-type *Anabaena* sp. strain PCC 7120 were analyzed by Southern blotting with a 1.4-kb *Acc*I-*Sca*I fragment containing the *icd* gene as the probe (Fig. 6A). The results obtained indicated that in strain MA1 the gene replacement had taken place, and an increase in size of 1.3 kb with respect to the wild-type hybridizing fragments was observed (Fig. 6B). However, only partial segregation of the inactivated version of the *icd* gene was achieved after several rounds of streaking. Since an *Anabaena icd* mutant was expected to be a glutamate auxotroph, like *icd* mutants in other organisms (i.e., *E. coli* and *Rhizobium* sp.), selection of the IDH$^-$ strain was done in BG11 medium supplemented with 0.5 mM glutamate because higher concentrations of this amino acid are toxic to *Anabaena* sp. (9). However, the K_m for L-glutamate transport of *Anabaena* sp. strain PCC 7120 is reported to be 0.5 mM (16), so to guarantee the complementation of the possible auxotrophy, we added proline to 3 mM. Proline is rapidly metabolized to glutamate in *Anabaena* sp. strain PCC 7120 (15a), but no significant difference in the level of segregation was observed between cells supplemented with amino acids and those not supplemented (Fig. 6B). We attempted to improve the segregation of the mutant by adding α-ketoglutarate to MA1 cultures. As shown in Fig. 6B, the segregation was favored by the presence of this ketoacid but some remaining copies of the wild-type *icd* gene were still present in the mutant strain, even when the interrupted gene was strongly selected by raising the neomycin concentration in MA1 cultures.

After several generations on medium containing 5 mM α-ketoglutarate, maximum segregation was reached and the mutant strain exhibited about 10% of the NADP$^+$-IDH activity present in wild-type *Anabaena* sp. strain PCC 7120, with either nitrate or ammonium as a nitrogen source and supplemented or not supplemented with the keto acid. Under N_2-fixing conditions, strain MA1 did not grow unless α-ketoglutarate was added to the culture.

When mutant strain MA1 was further cultivated on medium containing 5 mM α-ketoglutarate to obtain a completely segregated mutant, it failed to grow and became inviable. However, strain MA1 could be maintained in an intermediate state of segregation with 3 mM L-proline (Fig. 6B).

DISCUSSION

Characterization of NADP$^+$-IDH. In this report, we describe the first cloning and sequence analysis of a cyanobacterial *icd* gene. A detailed study of the purified NADP$^+$-IDH from the dinitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 is also presented.

The *Anabaena* NADP$^+$-IDH was purified by a procedure similar to that described for *Synechocystis* NADP$^+$-IDH (38); the main difference was the relative lability of *Anabaena* IDH in buffers not containing supplements of citrate, $MgCl_2$, and glycerol.

In general, both of the cyanobacterial IDHs purified so far are very similar. In regard to molecular structure, the *Anabaena* and *Synechocystis* NADP$^+$-IDHs belong to the first type of NADP$^+$-IDH defined by Chen and Gadal as dimers with apparently identical subunits with a molecular mass of 40 to 50 kDa (10). However, they have a molecular mass slightly higher (108 kDa) than those of most of the other well-characterized dimeric IDHs, such as that of *E. coli* (80 kDa). The kinetic and physicochemical parameters calculated for *Anabaena* NADP$^+$-IDH show a high degree of similarity to those of the *Synechocystis* enzyme; the most significant difference is the isoelectric point (5.5 for the *Anabaena* enzyme versus 4.4 for the *Synechocystis* enzyme) and therefore the relative mobility on nondenaturing gels (data not shown).

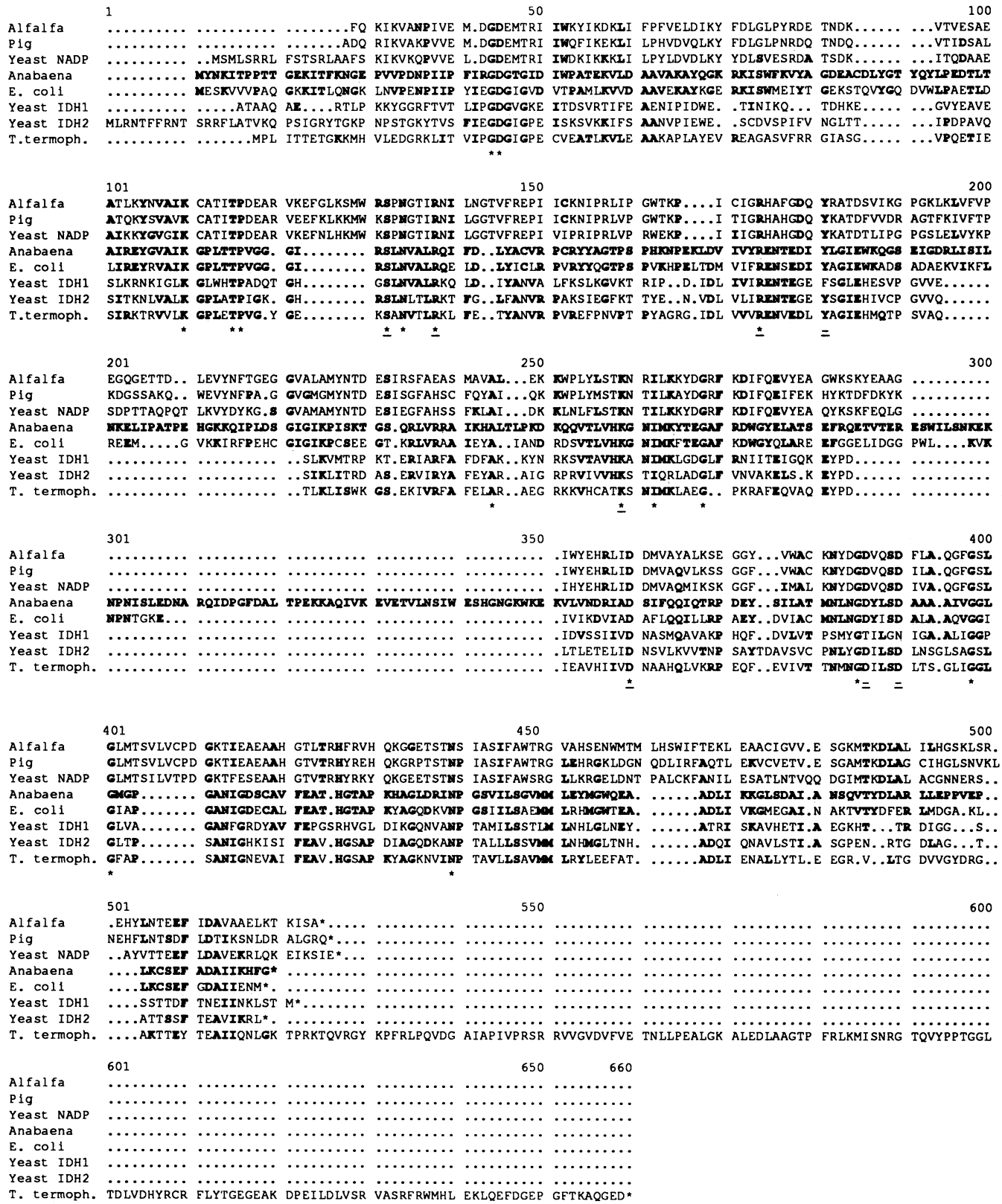

FIG. 5. Multiple alignment of prokaryotic and eukaryotic IDH sequences. Bold sequences represent those identical to the *Anabaena* NADP+-IDH sequence, asterisks denote identical amino acid residues in all of the proteins aligned, hyphens represent amino acid residues of *E. coli* IDH implicated in isocitrate binding that are conserved in all of the other sequences, and double hyphens denote those conserved in all of the sequences except the yeast IDH1 sequence. T. termoph., *T. thermophilus*.

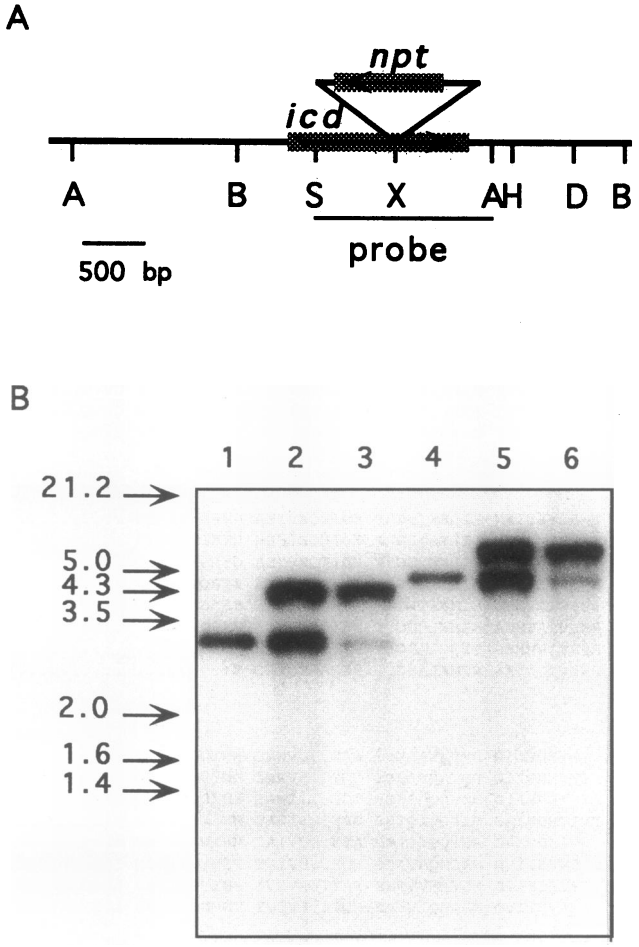

FIG. 6. Disruption construct and Southern blot analysis of the MA1 genomic disruption. (A) Structure of the *icd* region in wild-type *Anabaena* sp. strain PCC 7120. Insertion of an *npt* gene cassette at the location indicated generated mutant strain MA1. Restriction site abbreviations: A, *Acc*I; B, *Bst*XI; S, *Sca*I; X, *Xmn*I; H, *Hin*dIII; D, *Dra*I. (B) Southern blot analysis of DNAs from the wild-type strain (lanes 1 and 4) and strain MA1 cultivated for several generations with 3 mM L-proline (lanes 2 and 5) or 5 mM α-ketoglutarate (lanes 3 and 6) added to BG11 medium. Chromosomal DNAs from both strains were digested with *Acc*I (lanes 1 to 3) or *Dra*I (lanes 4 to 6) and hybridized to the 1.4-kb fragment marked "probe" in the restriction map. Size standards are indicated in kilobases.

The immunotitration of purified *Anabaena* $NADP^+$-IDH with antibodies raised against *Synechocystis* $NADP^+$-IDH, which do not cross-react with *E. coli* IDH, strongly suggests that the two cyanobacterial enzymes closely resemble each other.

Cloning and sequence analysis of the *icd* gene. By using a heterologous complementation strategy, we identified a 6-kb fragment of *Anabaena* DNA that rescues the glutamate auxotrophy of *icd* mutant *E. coli* DEK 2004. A similar strategy has recently been used to isolate the *Rhizobium meliloti icd* gene (34).

The high level of IDH activity in DEK 2004(pMA1) extracts, taken together with the electrophoretic mobility of the cloned enzyme and the immunological analysis, strongly argued that the gene cloned was *Anabaena icd*. In fact, the 1,419-bp open reading frame sequenced showed a high level of amino acid sequence similarity to the *E. coli* and *T. thermophilus* $NADP^+$-IDHs and *Saccharomyces cerevisiae* $NADP^+$-IDH, as well as with IMDHs from different sources.

Figure 5 shows a multiple alignment of several published IDH sequences. Two different IDH groups can be established from this sequence analysis on the basis of sequence similarity. One is formed by the $NADP^+$-IDHs of eucaryotic origin only, and the other includes the procaryotic IDHs and *S. cerevisiae* $NADP^+$-IDH subunits IDH1 and IDH2 (11, 12). The sequences of the first group, composed of the mitochondrial $NADP^+$-IDH from porcine heart (19) and *S. cerevisiae* (20) and an $NADP^+$-IDH from alfalfa (48), are more than 60% identical. The similarity in primary structure and cofactor specificity between these enzymes may suggest that their metabolic roles are also analogous. The sequences of the second group are more heterogeneous, and this group includes different cofactor specificities, as well as prokaryotic and eukaryotic sources; *E. coli* and *Anabaena* IDHs display the highest level of sequence identity (59%).

The *E. coli* enzyme has been extensively studied, and structural analysis by X-ray crystallography has identified the residues implicated in isocitrate and $NADP^+$ binding (24, 25). From the amino acid sequence comparisons of *E. coli* and *Anabaena* IDHs, some predictions about the possible role of individual residues can be made. With respect to the Mg-isocitrate complex, all of the residues directly related to the binding domain in *E. coli*, such as Ser-113, Arg-119, Arg-129, Arg-153, Tyr-160, Lys-230, Asp-283, Asp-307, and Asp-311, are conserved between the *E. coli* and *Anabaena* sequences and some of them (Ser-113, Arg-153, Lys-230, and Asp-283) are identical in all IDH sequences. Most of the residues participating in $NADP^+$ binding in *E. coli* are primarily clustered near the C terminus of the protein (Ile-37, Ile-320, Gly-321, His-339, Ala-342, Val-351, Asn-352, and Asp-392), and five of them (Gly-321, His-339, Ala-342, Asn-352, and Asp-392) are identical in *Anabaena* IDH. However, less conservation of these residues is apparent among all of the sequences aligned, indicating that the cofactor-binding domain is not as conserved as the isocitrate-binding domain.

The main difference between the *Anabaena* and *E. coli* IDH primary structures is an insertion of 44 amino acid residues in the C terminus of the *Anabaena* protein. In fact, a large gap needed to be introduced into the *E. coli* sequence to obtain the alignment shown in Fig. 5. This extra stretch (amino acid residues 286 to 329) is a hydrophilic region with a predicted α-helix secondary structure located within the small α/β domain (a typical α/β sandwich structure) described in the *E. coli* enzyme (26).

The amino acid sequence of *Anabaena* $NADP^+$-IDH also showed significant similarity to those of IMDHs from different sources. The fact that the sequence homology between cyanobacterial (*Anabaena*) IDH and (*Spirulina*) IMDH is not significantly higher than that found between *Anabaena* IDH and IMDHs from other sources suggests that these evolutionarily related enzymes diverged before the divergence of the cyanobacterial group.

The nucleotide sequence shown in Fig. 4 has a remarkable feature. Upstream of the *icd* gene, there are six imperfect copies of a 7-bp repeating sequence that has the consensus sequence CCCCAAT. This motif has also been found 3′ from *hetA* (22), at the 3′ end of *nifB*, and between *nifS* and *nifU* in *Anabaena* sp. strain PCC 7120 (37). Heptanucleotides with different consensus sequences (e.g., CAAATGA, CTAATGA, AGTCATT, and CCCCAGT) have been detected in heterocystous cyanobacteria near genes related to amino acid biosynthesis (17, 41), in the internal part of the RNA of RNase P (52)

or in the coding region of *nifJ* (2). The function of these repeats has not been determined, but it has been proposed that they might be the target of specific DNA-binding proteins (33), play a role in transcription (perhaps modulating the levels of downstream transcripts [37]), or play a role as a specific target for transposable elements or a hot spot for chromosome breakage in the DNA repair mechanism (2). The fact that these heptamer repeats are in some cases transcribed and translated, along with the lack of clear promoter sequences in the *icd* 5′ flanking region, could indicate that in our case these repeats are also transcribed, being the promoter of *icd* far away from this region. It may be significant that the heptanucleotides found upstream of *Anabaena* IDH have the same consensus sequence (CCCCAAT) as those found near genes involved in heterocyst differentiation (*hetA*) or nitrogen fixation (*nifBNSV* operon, *nifJ*), since IDH has been proposed to have a role in electron donation to nitrogenase (5, 27) (see below).

Metabolic role of NADP+-IDH. The fact that under the conditions used in this work, efforts to obtain a completely segregated *icd* mutant were unsuccessful strongly suggests that NADP+-IDH is essential for growth of *Anabaena* sp. strain PCC 7120. On the other hand, the gene cloned seems to be the only one encoding an IDH enzyme in this cyanobacterium.

NADP+-IDH is generally considered to be the enzyme responsible for supplying the α-ketoglutarate precursor for glutamate biosynthesis (10). In cyanobacteria, the IDH has the general role of providing the carbon skeletons needed for ammonium assimilation through the glutamine synthetase-glutamate synthase pathway (21). However, a potential physiological role of IDH as an electron donor to nitrogenase has also been proposed in different heterocystous cyanobacteria (5, 27). The pool of NADPH is linked through the ferredoxin: NADP oxidoreductase to ferredoxin, which serves as the immediate donor to nitrogenase (21).

The growth phenotype of strain MA1 (the partially segregated *icd* mutant), which is unable to grow on nitrogen-free medium without α-ketoglutarate, points to an essential role of IDH in the carbon skeleton provision for heterocysts; the level of IDH activity present in the mutant strain (10% of that of the wild type) seems not to be enough to maintain nitrogen assimilation in conditions of N_2 fixation. Since strain MA1 is able to grow at reduced rates on medium containing nitrate or ammonium as a nitrogen source, the α-ketoglutarate requirement must be higher on nitrogen-free medium, probably because ammonium assimilation is restricted to heterocysts under these conditions. This hypothesis agrees with the activity levels exhibited by the wild-type strain, which are higher under nitrogen-fixing conditions (38).

The fact that NADP+-IDH is essential for *Anabaena* growth may also be due to an NADPH requirement, since a completely segregated *icd* mutant cannot be obtained even when α-ketoglutarate is added to cultures. Another possibility for the essentiality of NADP+-IDH is that lack of this activity might lead to accumulation of toxic levels of citrate or isocitrate. In fact, it has been reported that *Rhizobium meliloti* and *E. coli icd* mutants spontaneously gave rise to mutants without citrate synthase activity (29, 34). If spontaneous selection of IDH^- CS^- double mutants does not take place in *Anabaena* sp., accumulation of a toxic product may be the reason for the lethal effect of *icd* disruption observed in this organism.

In conclusion, isocitrate toxicity or an α-ketoglutarate requirement leads to essentiality of NADP+-IDH activity in *Anabaena* sp. strain PCC 7120.

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