# Carbon Cycling in *Anabaena* sp. PCC 7120. Sucrose Synthesis in the Heterocysts and Possible Role in Nitrogen Fixation[1][OA]

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Nitrogen (N) available to plants mostly originates from $N_2$ fixation carried out by prokaryotes. Certain cyanobacterial species contribute to this energetically expensive process related to carbon (C) metabolism. Several filamentous strains differentiate heterocysts, specialized $N_2$-fixing cells. To understand how C and N metabolism are regulated in photodiazotrophically grown organisms, we investigated the role of sucrose (Suc) biosynthesis in $N_2$ fixation in *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120). The presence of two Suc-phosphate synthases (SPS), SPS-A and SPS-B, directly involved in Suc synthesis with different glucosyl donor specificity, seems to be important in the $N_2$-fixing filament. Measurement of enzyme activity and polypeptide levels plus reverse transcription-polymerase chain reaction experiments showed that total SPS expression is greater in cells grown in $N_2$ versus combined N conditions. Only SPS-B, however, was seen to be active in the heterocyst, as confirmed by analysis of green fluorescent protein reporters. SPS-B gene expression is likely controlled at the transcriptional initiation level, probably in relation to a global N regulator. Metabolic control analysis indicated that the metabolism of glycogen and Suc is likely interconnected in $N_2$-fixing filaments. These findings suggest that $N_2$ fixation may be spatially compatible with Suc synthesis and support the role of the disaccharide as an intermediate in the reduced C flux in heterocyst-forming cyanobacteria.

Nitrogen (N) is the fourth most abundant element in the biosphere. An estimated 80% to 90% of N available to plants in the terrestrial ecosystems originates from the biological conversion of nitrogen gas ($N_2$) to ammonia, an energetically expensive process linked to carbohydrate metabolism (Ludden and Barris, 1986). There are only a few prokaryotic microorganisms, including certain free-living or symbiotically associated bacteria and cyanobacteria that carry out $N_2$ assimilation. They do so through the action of the essentially anaerobic enzyme nitrogenase, an activity that requires reductants and ATP (Peters and Meeks, 1989; Crawford et al., 2000).

Diazotrophic cyanobacteria are the only organisms able to simultaneously and independently fix $N_2$ and produce photosynthetic molecular oxygen. Because nitrogenase is inhibited upon exposure to oxygen, different strains have adaptations that include either temporal or spatial separation of these processes (Berman-Frank et al., 2003). Some filamentous diazotrophic strains can differentiate a photosynthetic vegetative cell into a heterocyst through a variety of structural, biochemical, and genetic changes that allow active nitrogenase (Buikema and Haselkorn, 1991; Wolk, 2000; Yoon and Golden, 2001). To maintain a microaerobic environment, heterocysts have a thick envelope, an oxygen-producing deactivated PSII complex, and active respiration to scavenge any residual oxygen (Wong and Meeks, 2001).

Because heterocysts lack ribulose-1,5-diphosphate carboxylase, a key enzyme of the Calvin cycle, they are limited to heterotrophic metabolism and depend on vegetative cells for the generation of carbon (C) skeletons and reducing power (Wolk, 1968; Wolk et al., 1994; Zhang et al., 2006). The precise C compounds transported from vegetative cells into heterocysts remain to be definitively identified, although several carbohydrates, including Fru, erythrose, and Suc, have been suggested (Privalle and Burris, 1984; Schilling and Ehrnsperger, 1985). To elucidate the structure of the carrier molecule, Schilling and Ehrnsperger (1985) investigated the localization of Suc metabolism enzymes in *Anabaena variabilis*. They concluded that Suc synthase (SuS; EC 2.4.1.13) was responsible for Suc synthesis in vegetative cells, whereas alkaline invertase, which hydrolyzes Suc into hexoses, was suggested to be present almost exclusively in heterocysts. Based

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on this, it has been proposed that Suc might be the C transport molecule within the filament cells (Wolk et al., 1994).

Suc metabolism in *Anabaena* sp. PCC 7119 and PCC 7120 (also known as *Nostoc* sp. PCC 7120) has recently been elucidated. It has been demonstrated that Suc is synthesized through two different Suc-P synthases (SPS; EC 2.4.1.14) coupled to Suc-P phosphatase (SPP; EC 3.1.3.24). Suc can either be cleaved by SuS or irreversibly hydrolyzed by two alkaline/neutral invertases (A/N-Inv) when there is high demand for hexoses (Porchia and Salerno, 1996; Cumino et al., 2001, 2002; Curatti et al., 2002; Vargas et al., 2003). Curatti et al. (2002, 2006) showed SuS to be involved in the cleavage of Suc only in vegetative cells in vivo. Besides, in contrast to a previous report, it has recently been shown that A/N-Invs are also expressed in vegetative cells (Schilling and Ehrnsperger, 1985; Curatti et al., 2002; Vargas et al., 2003).

Studies on the relationship between C and N metabolism in heterocyst-forming cyanobacteria have focused on the role of glycogen in $N_2$ fixation (Ernst and Böger, 1985; Jensen et al., 1986; Ernst et al., 1990). During the light phase, most cyanobacteria strains accumulate a high level of glycogen, which is then mobilized to provide reductants and ATP during the night in either the vegetative cells or the heterocysts. $N_2$ fixation can, therefore, also take place at night, even if at a much lower rate (Fay, 1976; Lockau et al., 1978). Glycogen synthesis occurs through ADP-Glc donation of glucosyl for elongation of an $\alpha$-1,4-glucosidic chain. It is mainly regulated at the level of ADP-Glc synthesis catalyzed by ADP-Glc pyrophosphorylase (AGPase; EC 2.7.7.27), the enzyme encoded by the *agp* gene. AGPase activity was shown to be allosterically regulated by 3-phosphoglycerate (activator) and inorganic phosphate (Pi; inhibitor) in *Anabaena* sp. PCC 7120 (Ballicora et al., 2003). Whereas the role of glycogen in $N_2$ fixation has been examined, the role of Suc metabolism still remains to be understood.

The critical role of Suc in C flux modulation in the $N_2$-fixing filaments of *Anabaena* sp. was recently demonstrated by Curatti et al. (2002), who showed that diazotrophic growth was impaired in a mutant strain overexpressing SuS. Curatti et al. (2006) later showed that expression of SuS and Rubisco, a key enzyme in $CO_2$ fixation during photosynthesis, is similarly downregulated by a N source-dependent developmental program in the heterocysts.

The presence of two SPSs (SPS-A and SPS-B) with different glucosyl donor specificity (Porchia and Salerno, 1996; Cumino et al., 2002) raised the question of whether each may play a distinct role in Anabaena $N_2$-fixing filaments. We found that, whereas both SPSs contribute to Suc synthesis in vegetative cells, only SPS-B is active in heterocysts. Expression and metabolic analyses were integrated into a model in which a Suc cycle linked to glycogen metabolism and respiration may play a crucial role in the functional support of active heterocysts during the light.

## RESULTS

### SPS Expression Is Higher in Anabaena Cells Grown in $N_2$ Than in Combined N Conditions

To clarify the physiological role of Suc metabolism in heterocyst-forming cyanobacteria, we built on previous work analyzing the expression of enzymes involved in Suc biosynthesis. We showed that total SPS and SPP are only present in soluble protein fractions (Fig. 1) as reported in plants (Winter and Huber, 2000). Using permeabilized Anabaena filaments, we demonstrated that Suc synthesis occurs under light as well as dark conditions (Fig. 2A).

This study aimed to relate Suc synthesis to $N_2$ fixation. The experiments were performed in illuminated cells because most $N_2$ fixation takes place under light conditions (Stal, 2003). Analysis of the effect of N availability on SPS expression showed that total enzyme activity was maximal in $N_2$-fixing cells (Fig. 2, A and C). Immunoanalysis of the SPS polypeptide level showed a similar pattern of SPS expression (Fig. 2,

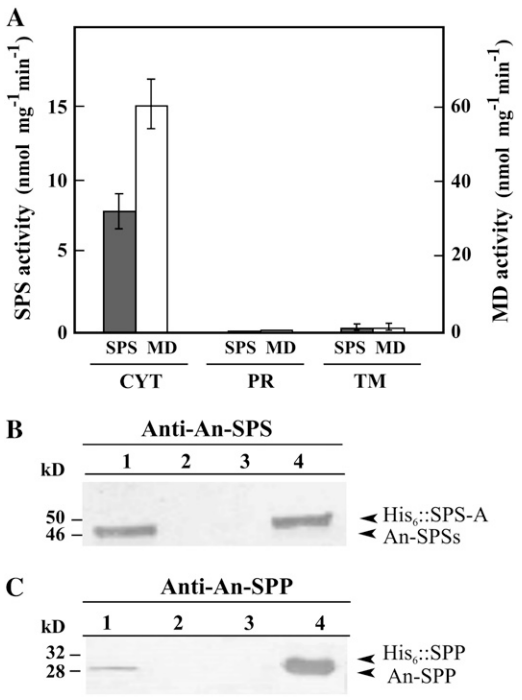


**Figure 1.** Subcellular localization of Suc biosynthesis enzymes in *Anabaena* sp. PCC 7120 cells. A, SPS activity from cytoplasmic (CYT), periplasmic (PR), and total membrane (TM) protein extracts. Malate dehydrogenase (MD) activity was used as the cytoplasmic marker. SPS activity was assayed in the presence of UDP-Glc. Data are the mean ± SE of five independent experiments. B and C, Immunoblotting of protein extracts. Lanes 1 to 3, CYT, PR, and TM, respectively. Lane 4, Control proteins ($His_6$::SPS-A [50 kD] or $His_6$::SPP [32 kD]). Polypeptides were revealed with anti-An-SPS (B) or anti-An-SPP (C). Approximately 200 and 1.5 $\mu$g of protein were loaded on lanes 1 to 3 and 4, respectively. Positions of molecular mass markers are indicated on the left (kD); arrowheads indicate the positions of native Anabaena SPSs (An-SPSs, approximately 46 kD), SPP (An-SPP, approximately 28 kD), and the recombinant proteins.

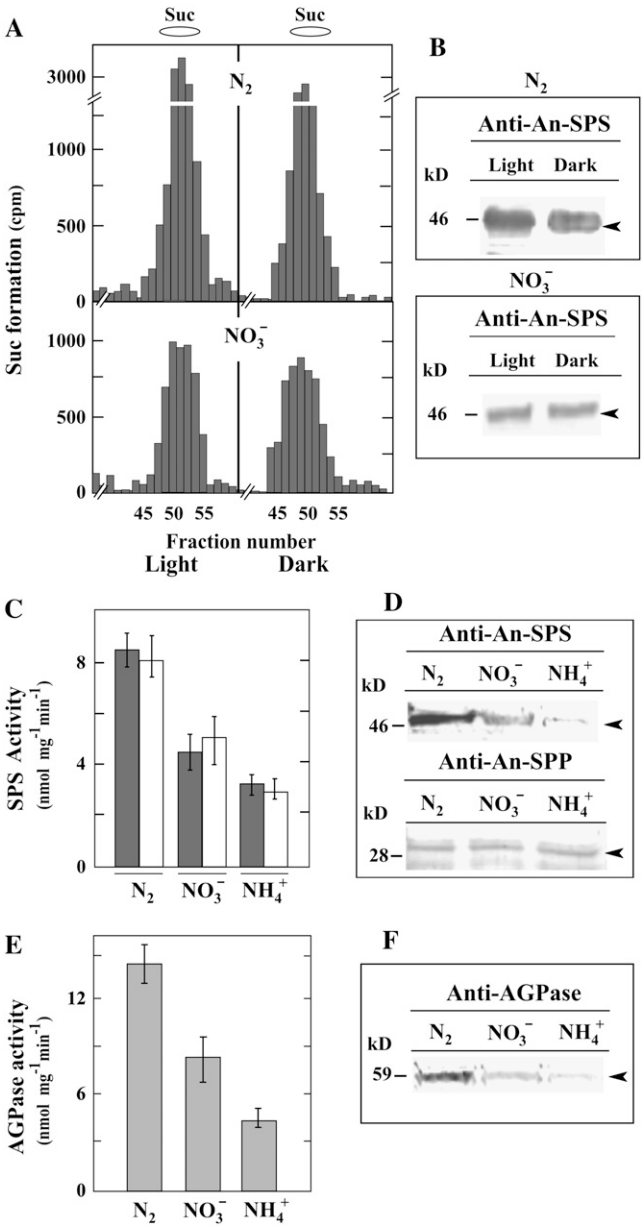


**Figure 2.** Effect of N source on SPS, SPP, and AGPase expression in *Anabaena* sp. PCC 7120 cells. A, Effect of light on the synthesis of Suc. Cells grown in $N_2$-fixing ($N_2$) or nitrate ($NO_3^-$) environment (top/bottom) were harvested during midlight or middark, permeabilized with toluene, and incubated with UDP-[U-$^{14}$C]Glc and Fru-6P. Labeled products were chromatographically separated and radioactivity determined in each fraction. The position of standard Suc is indicated at the top. B, Immunoblot analysis of proteins (100 $\mu$g/lane) from cells grown in $N_2$ (top) or $NO_3^-$ (bottom) revealed with anti-An-SPS. Cells were harvested in the midlight or middark. C to F, Cells grown in $N_2$, $NO_3^-$, or ammonium ($NH_4^+$) assimilation conditions and harvested during midlight. C, SPS activity assayed in the presence of UDP-Glc (dark gray bars) or ADP-Glc (white bars). Data are the mean ± SE of five independent experiments. D, Immunoblot analysis using anti-An-SPS (top, 100 $\mu$g/lane) or anti-An-SPP (bottom, 40 $\mu$g/lane). Positions of molecular mass markers indicated on the left (kD); arrowheads indicate position of Anabaena SPSs or SPP. E, AGPase activity. Data are the mean ± SE of three independent experiments. F, Immunoblot analysis (40 $\mu$g/lane) revealed with anti-An-AGPase.

B and D). Enzyme activity was also higher in the presence of ADP-Glc (SPS-B activity) in cells grown in a diazotrophic versus a N combined environment (Fig. 2C).

Transcriptional analysis of *spsA* and *spsB*, determined by reverse transcription (RT)-PCR, likewise showed maximal transcript level in N-deficient cells for both genes; the total amount in N-deficient cells was approximately 4 times higher than in the combined N-grown cells (Fig. 3). It was also found that *spsB* mRNA is likely to be predominant during $N_2$ fixation. Independent of the N source, the level of *sppA* transcripts was in accordance with the increase in *sps* gene expression (Fig. 3). SPP polypeptide levels, however, were similar in the different N sources provided in the medium (Fig. 2D).

In contrast to the high Suc-synthesizing capacity seen in $N_2$-fixing filaments, the Suc content of the N-2deficient-grown cells was approximately one-half that of the combined N-grown cells (6–10 nmol mg fresh weight$^{-1}$). Because both UDP-Glc and ADP-Glc can be substrates for Anabaena SPS reactions, expression of the enzymes responsible for their synthesis was also

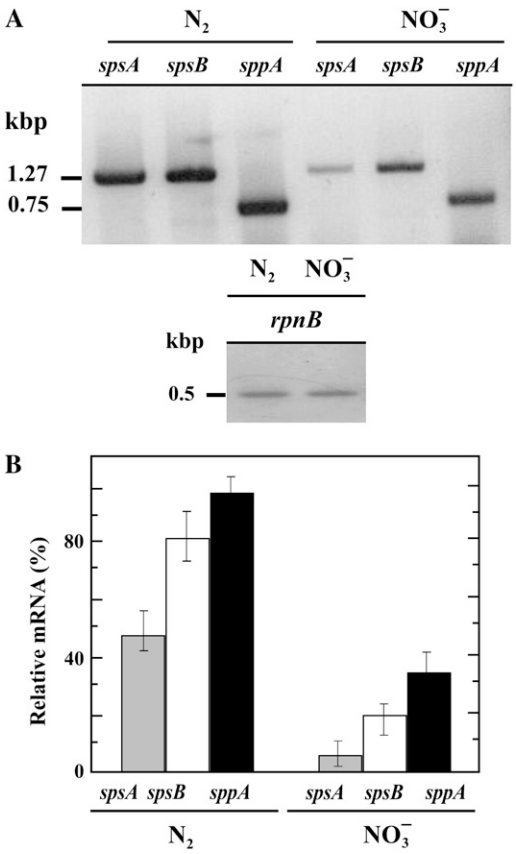


**Figure 3.** Expression of *spsA*, *spsB*, and *sppA* in *Anabaena* sp. PCC 7120 cells grown in $N_2$ or $NO_3^-$. A, RT-PCR analysis from total RNA. Amplification of Anabaena *rpnB* used as loading control. Position of molecular size markers indicated on the left. B, Densitometry of mRNA levels corresponding to detection in A. Values are the mean ± SE of five independent experiments.

**Table I.** *Steady-state properties of the kinetic model of Suc and glycogen metabolism of diazotrophically cultivated Anabaena sp. PCC 7120 cells*

| Reaction Name | $V_{max}$ | $K_m$ or $K_i$ | | Net Flux | Comment | Reference |
|---|---|---|---|---|---|---|
| | *nmol mg protein$^{-1}$ min$^{-1}$* | *mM* | | *nmol min$^{-1}$ mg fresh weight$^{-1}$* | | |
| **R1: Hexokinase** | 8.8 | $K_{m\ Glc}$ | 0.2 | 1.050 | Clamped concentrations of ATP and ADP | This study; Smith and Moore (1981) |
| Hexose + ATP → | | $K_{m\ Fru}$ | 7.5 | | | |
| hexose-P + ADP | | $K_{i\ ATP}$ | 1.0 | | | |
| **R2: SPS-A** | 2.8 | $K_{m\ Fru\text{-}6P}$ | 0.4 | 0.090 | Biosynthesis of Suc-6P | This study; Cumino et al. (2002) |
| UDP-Glc + Fru-6P → | | $K_{m\ UDP\text{-}Glc}$ | 1.3 | | | |
| Suc-6P + UDP | | $K_{i\ UDP}$ | 2.0 | | | |
| **R3: SPS-B** | 1.05 | $K_{m\ Fru\text{-}6P}$ | 2.5 | 0.490 | Biosynthesis of Suc-6P | This study; Cumino et al. (2002) |
| XDP-Glc + Fru-6P → | | $K_{m\ ADP\text{-}Glc}$ | 3.0 | | | |
| Suc-6P + XDP | | $K_{m\ UDP\text{-}Glc}$ | 4.0 | | | |
| (X = U or A) | | $K_{i\ ADP}$ | 4.0 | | | |
| **R4: SPP** | 4.4 | $K_{m\ Suc\text{-}6P}$ | 0.35 | 0.580 | Net production of Suc | Cumino et al. (2001) |
| Suc-6P → Suc + Pi | | $K_{i\ Suc}$ | 80 | | | |
| **R5: SuS** | 3.5 | $K_{m\ Fru}/K_{m\ UDP\text{-}Glc}$ | 52/2.7 | −0.090 | Flux is in direction of Suc cleavage | Porchia et al. (1999) |
| XDP-Glc + Fru → | 0.7 | $K_{m\ Fru}/K_{m\ ADP\text{-}Glc}$ | 4.2/1.3 | | | |
| Suc + XDP | 1.8 | $K_{m\ Suc}/K_{m\ UDP}$ | 303/1.25 | | | |
| (X = U or A) | 0.7 | $K_{m\ Suc}/K_{m\ ADP}$ | 305/1.15 | | | |
| **R6: INV** | 1.8 | $K_{m\ Suc}$ | 10 | 0.295 | Irreversible Suc hydrolysis | Vargas et al. (2003) |
| Suc + water → | | | | | | |
| Glc + Fru | | | | | | |
| **R7: Hexose-P mutase** | 2.8 | $K_{m\ Glc\text{-}1P}$ | 0.2 | −0.490 | Flux is in direction of Glc-1P production | Pearce and Carr (1969) |
| Glc-1P → Glc-6P | | | | | | |
| **R8: Hexose-P isomerase** | 4.2 | $K_{m\ Fru\text{-}6P}$ | 0.25 | 0.190 | Flux is in direction of Fru-6P production | Pearce and Carr (1969) |
| Glc-6P → Fru-6P | | $K_{m\ Glc\text{-}6P}$ | 5 | | | |
| **R9: UGPase** | 52 | $K_{m\ Glc\text{-}1P}/K_{m\ UTP}$ | 0.1/0.3 | 0.000 | The mass action ratio equals the $K_{eq}$ and the change in free energy is zero at steady state. Under these conditions, the net flux is null. | This study |
| UDP-Glc + PPi → | 130 | $K_{m\ UDP\text{-}Glc}/K_{m\ PPi}$ | 0.1/0.2 | | | |
| Glc-1P + UTP | | | | | | |
| **R10: AGPase** | 7.8 | $K_{m\ Glc\text{-}1P}/K_{m\ ATP}$ | 0.07/0.1 | | | |
| ADP-Glc + PPi → | 24 | $K_{m\ ADP\text{-}Glc}/K_{m\ PPi}$ | 0.4/0.03 | −0.490 | Flux is in direction of ADP-Glc production, involved in glycogen and Suc biosynthesis | Ballicora et al. (2003) |
| Glc-1P + ATP | | | | | | |

investigated. It was seen that UDP-Glc pyrophosphorylase (UGPase; EC 2.7.7.9) activity was similar in cells grown in $N_2$ and in combined N (approximately 55 nmol UDP-Glc min$^{-1}$ mg$^{-1}$), whereas a higher AGPase activity was seen in $N_2$-fixing cells (Fig. 2E). A similar expression pattern was noted in polypeptide level and mRNA content (Fig. 2F; data not shown).

### Modeling the Suc Network in $N_2$-Fixing Anabaena Filaments

To contribute to an understanding of the interconnection between Suc synthesis and the presence of nucleoside diphosphates, we performed product inhibition assays for the two Anabaena SPSs and modeled C flux under diazotrophic conditions. The formal model was built using the Gepasi framework (Mendes, 1997). The simulation included information from experimental kinetic data obtained in this study from *Anabaena* sp. in conjunction with values reported in the literature.

Involvement of the two SPSs in Suc production and the relationship between Suc and glycogen metabolism in Anabaena cells diazotrophically grown in the light were investigated through metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Because this study could provide a quantitative depiction of C flow through the two competing metabolic pathways, we included the main reactions of both

metabolisms (Fig. 4). The evaluated fluxes are shown in Table I and Figure 4. Fluxes were designated as positive when their net direction was forward (left to right in the equation in Table I) and negative when their direction was backward (right to left in the equation).

In all cases, the fluxes and steady-state concentrations of the variable metabolites calculated by the model were in agreement with experimental determinations. Negative fluxes were observed for SuS, hexose-P mutase, and AGPase. This indicated that the reactions catalyzed by these enzymes may operate in vivo in a direction opposite to that outlined in Table I. The flux through SuS should have been toward the cleavage of Suc and the flux through hexose-P mutase toward the formation of Glc-1P, a substrate for the nucleoside-triphosphate-hexose-1P nucleotidyltransferases (AGPase and UGPase). The UGPase reaction operated close to equilibrium, as consistent with previous findings (Roscher et al., 1998; Kleczkowski et al., 2004). The simulation showed the SPS-B flux (0.490 nmol $min^{-1}$ mg fresh $weight^{-1}$) to be approximately 5 times higher than the SPS-A flux. The sum of the two fluxes agrees with SPP flux, accounting for the entire Suc biosynthesis capacity.

SPS-A was inhibited by UDP (approximately $K_{i\ UDP}$ of 2.0 ± 0.5 mM). This supports the hypothesis that the preferred substrate for this enzyme is UDP-Glc. On the other hand, SPS-B was inhibited only by ADP, whether UDP-Glc or ADP-Glc (approximately $K_{i\ ADP}$ of 1.8 ± 0.3 and 3.9 ± 0.5 mM, respectively) served as the glucosyl donor. When the SPS-A reaction was removed from the model, the net flux through SuS was interrupted ($-5 \times 10^{-11}$ nmol $min^{-1}$ mg fresh $weight^{-1}$), whereas the fluxes through invertases and UGPase remained unaltered (0.350 and $6 \times 10^{-11}$ nmol $min^{-1}$ mg fresh $weight^{-1}$, respectively).

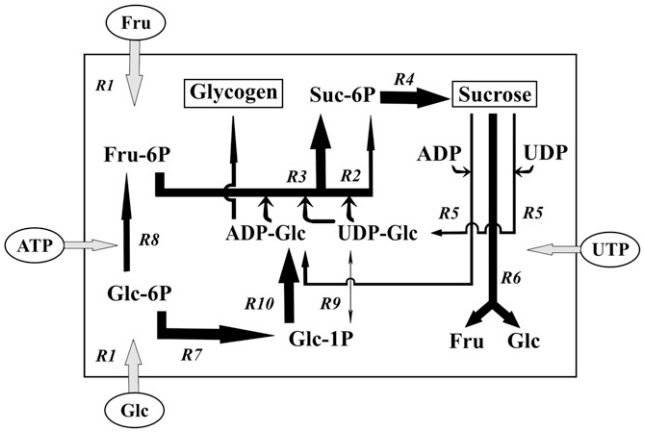


**Figure 4.** Metabolic flux map of primary and intermediate reactions involved in Suc and glycogen metabolism in a model of illuminated $N_2$-fixing Anabaena filaments. Arrow widths are proportional to flux values; arrowhead indicates direction of net flux. Each flux is assigned for a number of reactions (R1–R10) indicated in Table I. The intracellular space contained is framed. Metabolites (Fru, Glc, ATP, and UTP) taken up from the extracellular medium are shown in ovals and sink final products (glycogen and Suc) in rectangles.

### Differential Expression Patterns of *spsA* and *spsB* in Anabaena $N_2$-Fixing Filaments Show That Only SPS-B Is Involved in Heterocyst Metabolic Activity

Immunoblot analysis and enzyme activity measurements showed that SPS is present not only in the vegetative cells, but also in the heterocysts of two Anabaena strains (Fig. 5). This was confirmed by incorporation of labeled C into Suc from UDP-[U-$^{14}$C]Glc (approximately 3,000 cpm $min^{-1}$ mg fresh $weight^{-1}$) or ADP-[U-$^{14}$C]Glc (approximately 2,500 cpm $min^{-1}$ mg fresh $weight^{-1}$) into permeabilized heterocysts in *Anabaena* sp. PCC 7120 in the presence of Fru-6P. Furthermore, only *spsB* transcripts, which are accompanied by *sppA* expression, were detected in the heterocysts (Fig. 6A).

The localization of SPS-A and SPS-B in $N_2$-fixing filament cells was also evidenced with transcriptional fusions of an optimized version of the green fluorescent protein (GFP) gene (*gfp*-mut2) to putative promoters of *spsA*, *spsB*, and *sppA* ($P_{spsA}$, $P_{spsB}$, and $P_{sppA}$, respectively). DNA fragments of 950, 477, and 559 bp upstream from the translation start site of *spsA*, *spsB*, and *sppA*, respectively, directed the expression of *gfp*-mut2 in *Anabaena* sp. PCC 7120 (Fig. 6B). In diazotrophically grown Anabaena cells, *spsA* expression was detected only in vegetative cells (Fig. 6C, a–c), whereas *spsB* and *sppA* expression was localized to both vegetative cells and heterocysts (Fig. 6C, d–i). *Anabaena* sp. PCC 7120 cells harboring a plasmid (pAM1000) in which a heterocyst cell-specific promoter ($P_{nifHDK}$) was fused to *gfp* is shown as control (Fig. 6C, j–l). No fluorescence signal was detected in Anabaena cells containing a control plasmid (pAMpmt$^-$) carrying a promoterless *gfp* construct (data not shown).

### Transcriptional Regulation of *spsB* under $N_2$ Fixation

The finding that only *spsB* is expressed in the heterocysts of $N_2$-fixing filaments led to investigation of possible mechanisms for its transcriptional regulation. This was undertaken through determination of the RNA 5′ ends that correspond to the transcription start points (tsps), mapped by primer extension of RNA obtained from *Anabaena* sp. PCC 7120 grown under different N conditions. Two RNA sizes ($RNA_I$ and $RNA_{II}$) were observed, starting at the −54 (tspI) and −93 (tspII) nucleotides upstream of the translation initiation site (Fig. 7). Each tsp was confirmed through similar experiments conducted with two different oligonucleotides. $RNA_I$ and $RNA_{II}$ were detected under the three N culture conditions assayed, but *spsB* expression was greater in cells grown in $N_2$ than in combined N (Fig. 7A). The extension product of $RNA_{II}$ was, however, 4 times more abundant than that of $RNA_I$ in $N_2$-fixing cells. Analysis of the upstream sequences of the two putative promoters showed a −10 box similar to those of *Escherichia coli* $\sigma^{70}$ promoters

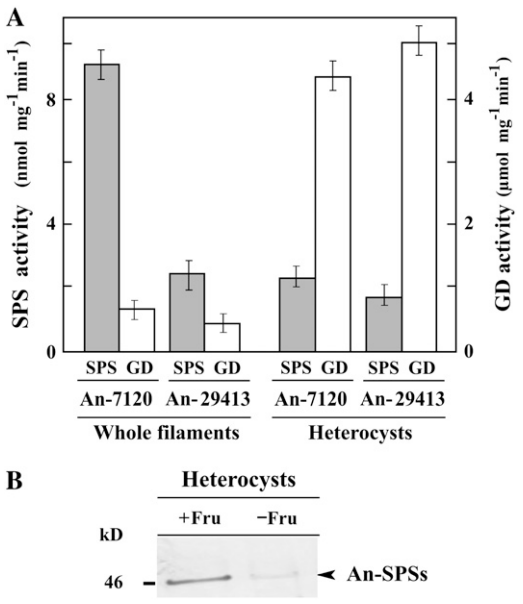


**Figure 5.** SPS expression in two Anabaena strains grown in $N_2$-fixing conditions. A, SPS activity (gray bars) assayed in whole filaments or heterocyst extracts of *Anabaena* sp. PCC 7120 (An-7120) and *A. variabilis* ATCC 29413 (An-29413). Glc-6P dehydrogenase (GD) activity (white bars) was included as a heterocyst marker. Results are the mean ± SE ($n = 5$). B, Immunoblot analysis of SPS expression from heterocyst extracts of Anabaena cells grown in N-deprived conditions with (+Fru) or without (−Fru) 10 mM Fru.

(Fig. 7B). Because *spsB* expression seemed to be regulated by the N source, we went on to investigate in the two putative promoters the possible presence of putative binding sites of N control A (NtcA), a DNA-binding protein involved in the control of transcription of N-regulated genes in cyanobacteria (Herrero et al., 2004). Similar to the consensus sequence motif GTA(N8)TAC described for $N_2$-activated genes (Herrero et al., 2001), the sequence GTA(N8)ACA was found at −39 with respect to tspII (Fig. 7, B and C).

## DISCUSSION

A close relationship between Suc metabolism and the $N_2$ fixation process has been described in legume-Rhizobium symbiosis. Degradation of Suc imported from the plant by SuS is a key initial step in the development and normal function of the nodule and maintenance of nitrogenase activity (Gordon et al., 1999). Suc biosynthesis, however, is unlikely to take place in the heterotrophic nodule. A different scenario of $N_2$ fixation is the filament of diazotrophic cyanobacteria, where Suc metabolism has been described (Curatti et al., 2002) and Suc cleavage takes place only in photosynthetic vegetative cells (Curatti et al., 2006). This study focused on the fact that Suc biosynthesis occurs in $N_2$-fixing filaments of *Anabaena* sp. PCC 7120, modeling the C flux between Suc and glycogen. The presence of an active SPS in the heterocysts (SPS-B) was uncovered in addition to a specific isoform in the vegetative cells (SPS-A), both of which relate to the dynamic mechanism underlying $N_2$ fixation.

Although Suc biosynthesis is higher in diazotrophically grown filaments than in cells grown under combined N conditions (Figs. 2 and 3), a very small amount of Suc accumulation is detectable due to a high rate of disaccharide degradation (Curatti et al., 2002; Vargas et al., 2003; Table I). This notable finding of Suc turnover may provide additional support for the role of Suc as an intermediate in the flux of reduced C in the $N_2$-fixing filament (Wolk et al., 1994; Curatti et al., 2002). However, contrary to previous reports (Schilling and Ehrnsperger, 1985), we found that Suc biosynthesis can take place not only in vegetative cells, but also in heterocysts. Unlike plant root nodules, heterocysts are able to synthesize the disaccharide (Figs. 5 and 6), as well as import C from adjacent cells, as was similarly described to occur in heterotrophic plant tissues (Roscher et al., 1998; Babb and Haigler, 2001; Im, 2004).

Whereas the presence of one SPS gene is likely to be characteristic of unicellular cyanobacterium strains, filamentous $N_2$-fixing cyanobacteria were reported to have two genes (*spsA* and *spsB*) present (Curatti et al., 1998; Cumino et al., 2002; Salerno and Curatti, 2003). Each gene, however, is transcribed in Anabaena independently of the $N_2$ fixation process. The two genes are simultaneously expressed in diazotrophically and combined N-grown vegetative cells (Figs. 3 and 6). The possibility that the two SPS isoforms fulfill distinct metabolic functions was explored by examining the distribution of Suc biosynthesis genes in the *Anabaena* sp. PCC 7120 genome. Interestingly, *spsA* and *sppA* are located in the same genome half (the 265°–85° region); meanwhile, *spsB* is found in the other genome half (85°–265° region), reported by Sugaya et al. (2004) to contain most of the protein genes performing housekeeping functions essential to cyanobacteria and genes that code for proteins with functions needed in a particular environment (such as for $N_2$ fixation and metabolism under N deprivation conditions, respectively).

Because control of *spsB* expression may occur at transcriptional initiation (Fig. 7), primer extension experiments were conducted. These indicated that the putative *spsB* promoter inferred from tspII shares a sequence similar to that of promoters activated by NtcA. The latter is a cAMP receptor protein family transcription factor, which, in the absence of ammonium, promotes the expression of alternative N source assimilation genes (Herrero et al., 2001). Suc biosynthesis may thus be coordinated with N assimilation by NtcA. SPS-B, similarly to the products of NtcA-activated genes, may be involved in heterocyst differentiation, development, and function (Herrero et al., 2004). Transcriptional regulation and the absence of allosteric modulation by Glc-6P and Pi are likely to be a particular feature of cyanobacterial SPSs (Porchia and Salerno, 1996; Curatti et al., 1998; Desplats et al., 2005).

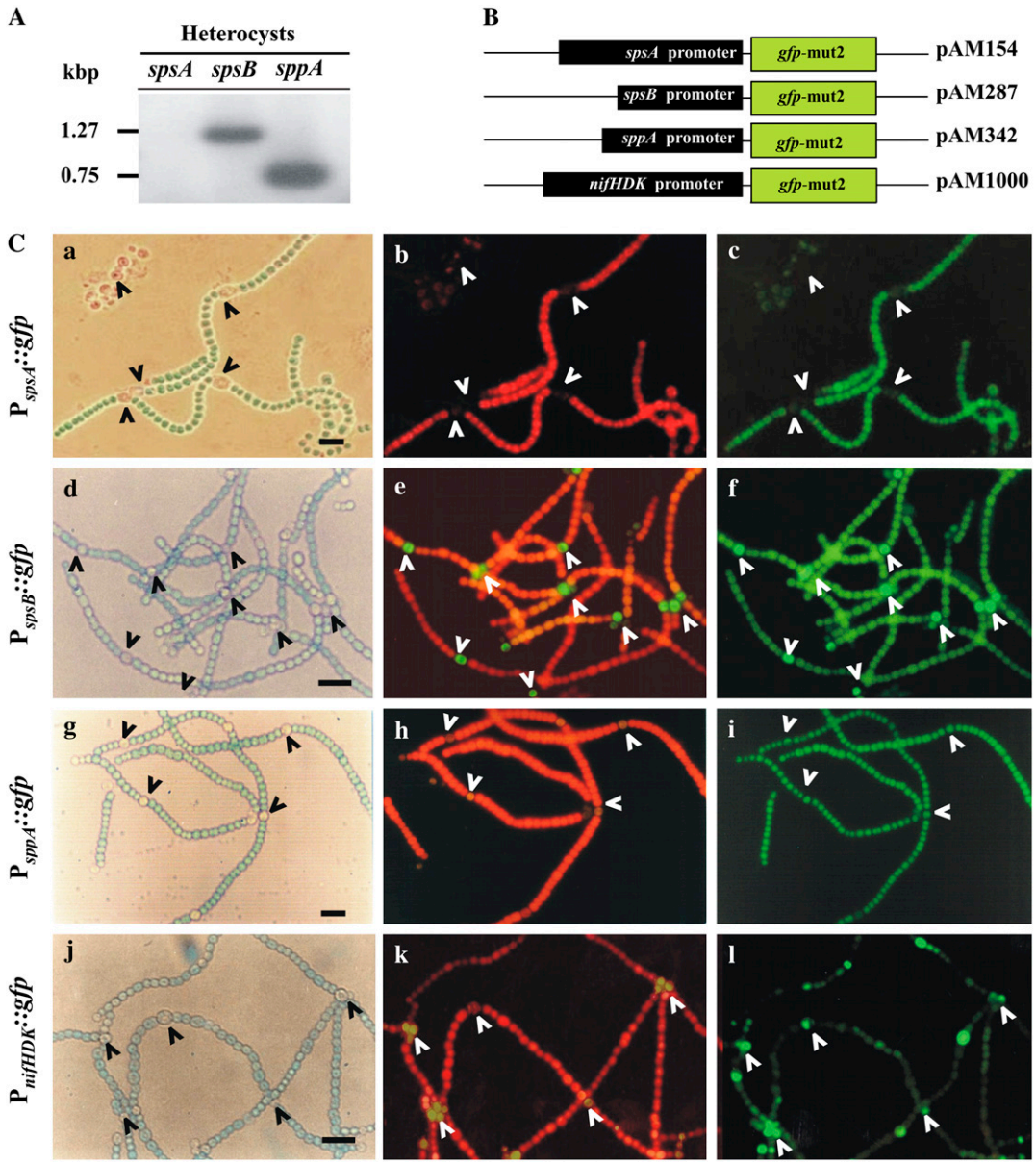


**Figure 6.** Cellular localization of *spsA*, *spsB*, and *sppA* expression in $N_2$-fixing filaments of *Anabaena* sp. PCC 7120. A, RT-PCR analysis from total heterocyst RNA. B, Schematic representation showing a physical map of the GFP fusion constructs. The *gfp* sequence was fused in frame with upstream sequences of the translational start codon of *spsA*, *spsB*, *sppA*, and *nifHDK*. C, Cellular expression of $P_{spsA}$::*gfp*, $P_{spsB}$::*gfp*, and $P_{sppA}$::*gfp* reporter transcriptional fusion in *Anabaena* sp. PCC 7120 filaments subjected to prolonged diazotrophic growth. Each horizontal set of photographs corresponds with the same microscopic field. Contrast photomicrographs were obtained in full-spectrum light (a, d, g, and j). Chlorophyll fluorescence micrographs were taken without the emission filter (b, e, h, and k). GFP fluorescence micrographs were obtained with the corresponding emission filter (c, f, i, and l). Arrowheads indicate heterocysts. All microphotos taken at the same magnification (1,000×). Images photographed and processed with same settings to allow qualitative comparison of fluorescence intensities. Scale bars, 10 $\mu$m.

Experimental data from this study point to the interconnection between glycogen and Suc metabolism in Anabaena $N_2$-fixing filaments. Glycogen is produced and stored only during the day, serving as the predominant metabolic fuel at night (Stal and Moezelaar, 1997), but Suc is biosynthesized in both light or darkness (Fig. 2, A and B). Glycogen may, therefore, be the C source for disaccharide formation at night. Chen et al. (2005) recently reported that SPS performs a crucial function in plants in synthesizing Suc during starch mobilization at night. On the other hand, the considerable accumulation of glycogen in Anabaena during $N_2$ fixation in the light (Ernst and Böger, 1985) is in accordance with a high level of AGPase activity

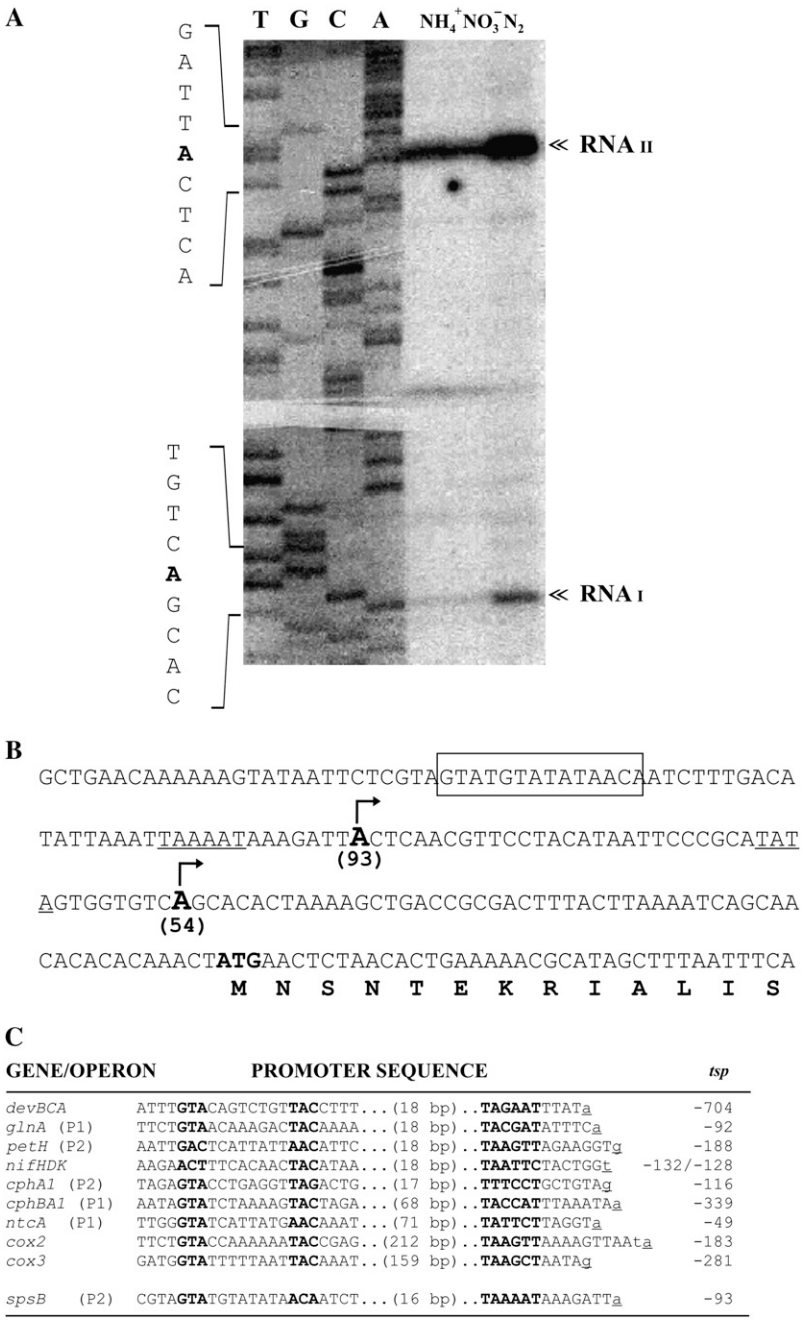


**Figure 7.** Origins of *spsB* transcription and analysis of the putative promoter regions. A, Primer extension mapping of the tsps of *spsB* carried out with total RNA (30 $\mu$g) from Anabaena cells grown in different N sources and the oligonucleotides B-*ol*-tsp3. A similar result was obtained with B-*ol*-tsp4 (data not shown). The sequencing ladders presented (lanes T, G, C, and A) were generated with the same primers used in the primer extension reactions. Arrowhead points to the extension product identifying the putative tsps. B, Nucleotide sequence of the *spsB* upstream region. The tsps (arrows) and start translation codon are in bold. Numbers in parentheses indicate the relative position from the translation start. A sequence similar to the consensus NtcA-binding sequence is boxed. The −10 box regions are underlined. C, Sequence alignments of NtcA-activated promoters (P) whose products act during differentiation and in the mature Anabaena heterocyst (*devBCA*, ATP-binding cassette transporter; *glnA*, Gln synthetase; *petH*, ferredoxin-NADP reductase; *nifHDK*, nitrogenase complex; *cphA1*, cyanophycin synthetase; *cphBA1*, cyanophycinase; *ntcA*, N regulator; *cox2* and *cox3*, terminal respiratory oxidases). Location of the tsps with respect to the tsp of the corresponding gene is indicated. The consensus for NtcA-binding site and −10 hexamers is in bold.

(Fig. 2, E and F). This study, however, found that AGPase flux alone, as calculated by metabolic simulation, is insufficient to supply the ADP-Glc needed for glycogen synthesis and Suc production through SPS-B (Table I; Fig. 4). A concomitant production of ADP-Glc might also be ascribed to Suc cleavage by SuS in the vegetative cells (Table I) in keeping with this enzyme's reported role (Porchia et al., 1999; Curatti et al., 2002, 2006). A null flux through SuS when the SPS-A reaction was removed from the metabolic model was also seen, leading us to speculate that the activity of the two enzymes may be connected in the vegetative cells of the $N_2$-fixing filament. Building on the recent report by Curatti et al. (2006), our findings suggest that *susA* and *spsA* may be similarly down-regulated in the developmental heterocyst differentiation program.

Suc synthesis in the heterocyst could be paralleled to Suc synthesis in heterotrophic plant tissues. The heterocyst, however, is very distinct from cotton (*Gossypium hirsutum*) fibers, etiolated hypocotyls, or germinating seeds, for example, in which Suc pool regulation is modulated by simultaneous degradation and resynthesis and Suc cleavage by SuS plays a central role (Geigenberger and Stitt, 1991; Geigenberger et al.,

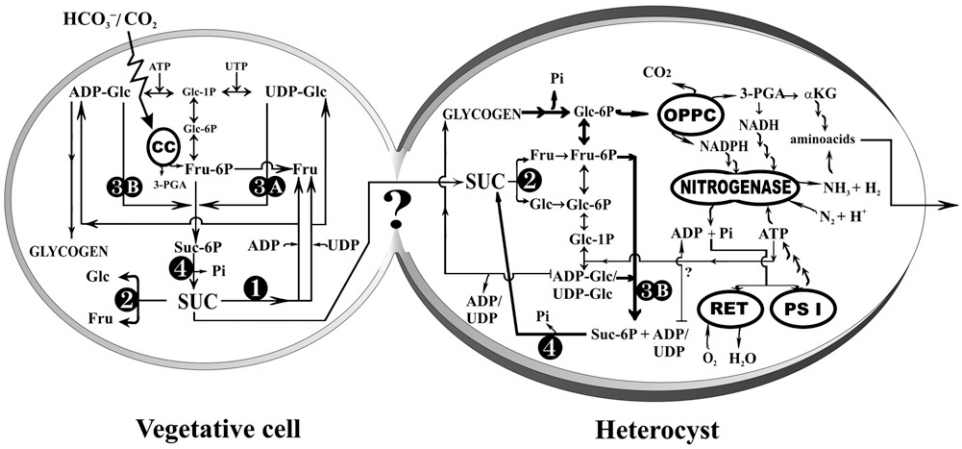


**Figure 8.** Schematic diagram of Suc pathway in $N_2$-fixing Anabaena filament cells. Suc enzyme localization in *Anabaena* sp. PCC 7120 illuminated filaments based on this study and Curatti et al. (2006). Photosynthetic C fixation through the Calvin cycle (CC) occurs in the vegetative cells and could lead to Suc and glycogen biosynthesis. Heterocysts act as an important sink for carbohydrates from vegetative cells and as a source of fixed N (Wolk et al., 1994). In heterocysts, which could also synthesize glycogen and Suc, the reductants for $N_2$ and $O_2$ reduction are generated by the activity of the oxidative pentose-P cycle (OPPC), the NADPH heterocyst-specific ferrodoxin, and respiratory electron transport (RET), as well as the ATP synthesis by cyclic phosphorylation (PSI). Suc enzymes are indicated as (1) SuS; (2) A/N-Inv; (3A) SPS-A; (3B) SPS-B; and (4) SPP. αKG, α-Ketoglutarate.

1999; Nguyen-Quoc and Foyer, 2001). As Anabaena heterocysts lack SuS activity (Curatti et al., 2006), Suc degradation occurs solely via A/N-Inv, which Böhme (1998) proposed necessary for C supply, maintenance of a high respiration rate, and provision of the C acceptors for ammonia required for $N_2$ fixation.

Substrate cycling involving carbohydrate turnover in plant tissues has been widely reported, but its mechanisms and functions remain poorly understood (Alonso et al., 2005). Our results suggest that a Suc-cycling mechanism may be operating in heterocysts, allowing cell metabolism to shift easily from Suc production to degradation, through A/N-Inv, hexokinase, hexose-P mutase, hexose-P isomerase, AGPase, SPS-B, and SPP activity. Although the precise metabolic role of the Suc cycles involving SPS/Inv has not yet been determined, several functions in plants have been proposed (Roscher et al., 1998; Geigenberger et al., 1999). It has been suggested that Suc cycles allow a pathway's net flux a high degree of sensitivity to respond to factors modulating rates of synthesis and degradation, controlling respiration, maintaining osmotic potential, controlling sugar accumulation, and promoting sugar signaling (Rohwer and Botha, 2001; Roby et al., 2002). Whereas we have been able to demonstrate the operational feasibility of Suc cycles, further work will be needed to elucidate Suc cycle function in the heterocyst.

Our findings are depicted in the model in Figure 8. Suc translocated from the vegetative cells into the heterocysts may be the respiratory substrate (Wolk et al., 1994; Herrero et al., 2004), after hydrolysis to Glc and Fru by a high level of invertase activity (Schilling and Ehrnsperger, 1985; Curatti et al., 2002). The resulting hexoses, after phosphorylation by hexokinases, can enter the oxidative pentose-P cycle and respiration to provide NADPH and C skeletons for ammonium assimilation and act as glycogen synthesis precursors. Suc resynthesis by SPS-B may take place in the heterocysts, likely from glycogen degradation products, contributing to an operational Suc-cycling mechanism. It may be that SPS-B is contributing to the ADP pool and thus to the high ADP-ATP ratio reported in Anabaena heterocysts (Privalle and Burris, 1983) and to modulate nitrogenase activity through the inhibition of proteolytic degradation of the dinitrogenase reductase subunit (Durner et al., 1996).

Ongoing work is focusing on creating *spsA* and *spsB* mutant strains to further pinpoint the role of Suc biosynthesis in the $N_2$-fixing filaments. Future studies on the role of Suc during the early and later stages of heterocyst differentiation should help to bring some light on the physiological role of Suc in the N source-dependent development of these specialized cells.

## MATERIALS AND METHODS

### Biological Material and Culture Conditions

Axenic cultures of *Anabaena* sp. strain PCC 7120 and *Anabaena variabilis* ATCC 29413 were grown photoautotrophically under a 12-h light/12-h dark cycle with white fluorescent light at 30°C with orbital shaking. Media used for growth were BG11 (prepared with $KNO_3$ instead of $NaNO_3$), $BG11_0$ (BG11 medium lacking nitrate), and $BG11_0NH_4^+$/TES [BG11 medium lacking nitrate and supplemented with 2.5 mM $NH_4Cl$ and 5 mM *N*-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES-KOH buffer, pH 7.5); Curatti et al., 2002]. Cells were harvested at the late-exponential phase by centrifugation during the midlight or middark period, washed, and either stored at −80°C or toluene permeabilized. For strains containing the $P_{spsA}$::*gfp*, $P_{spsB}$::*gfp*, and

$P_{nifHDK}$::*&fp* transcriptional reporters (plasmids pAM154, pAM287, and pAM1000, respectively), neomycin at 25 and 100 $\mu$g $mL^{-1}$ was added to liquid and solid media, respectively (Yoon and Golden, 2001).

*Escherichia coli* strains were grown at 37°C in Luria-Bertani liquid or agar medium supplemented with appropriate antibiotics according to standard protocols (Sambrook and Russell, 2001). *E. coli* BL21(λDE3):pLysS strain (Novagen) was used for the overproduction of SPS-A and SPP proteins of *Anabaena* sp. PCC 7120, as previously reported by Cumino et al. (2001, 2002).

## Cell Fractionation and Heterocyst Isolation

Membrane and periplasm proteins were purified from 2 L of *Anabaena* sp. PCC 7120 cultures, according to Norling et al. (1998) and Zhu et al. (1999), respectively. Heterocysts were isolated from diazotrophically grown cells of *A. variabilis* sp. ATCC 29413 and *Anabaena* sp. PCC 7120, according to Zhou et al. (1998), with or without Fru in the medium (Jensen et al., 1986). Heterocyst preparations were monitored by light microscopy and used once heterocyst purity was approximately greater than 95% of the total cell number. Quantification of proteins was done using Bradford's dye-binding assay.

## Protein Extraction and Immunoblot Analysis

Total cell extracts from Anabaena vegetative cells and heterocysts were prepared as previously reported (Cumino et al., 2001) and desalted through Sephadex G-50 columns. Proteins were separated by SDS-PAGE using a MiniProtean system (Bio-Rad) and five-well combs (0.75 mm) on 12% or 15% polyacrylamide gels for SPS/AGPase and SPP analyses, respectively (Cumino et al., 2001). Polypeptides were visualized with Coomassie Blue or electroblotted onto nitrocellulose membranes (HyBond C; Amersham), which were probed with the polyclonal antibodies anti-An-SPS, anti-An-AGPase, or anti-An-SPP and raised in rabbits against $His_6$::SPS-A (Cumino et al., 2002), $His_6$::AGPase (this study), or native SPP purified from Anabaena cells (Cumino et al., 2001), respectively.

## Enzyme Assays

Because SPS-A and SPS-B show different substrate specificity (both SPSs accept UDP-Glc as the glucosyl donor, but only SPS-B uses ADP-Glc), total SPS activity was assayed in a reaction mixture containing Fru-6P and UDP-Glc, and SPS-B activity was carried out in the presence of ADP-Glc (Porchia and Salerno,1996). SPS-B catalytic efficiency with ADP-Glc is twice that with UDP-Glc (Porchia and Salerno, 1996). SuS, SPP, and A/N-Inv activity were measured as previously reported (Porchia et al., 1999; Cumino et al., 2001; Vargas et al., 2003). SPP activity could not be measured in crude extracts, as unspecific phosphatases interfered with the assay. Glc-6P dehydrogenase activity, used as an enrichment marker in heterocyst preparation (30- to 60-fold higher specific activity in heterocysts versus vegetative cells), was estimated by monitoring the Glc-6P-dependent increase in NADPH absorbance (Summers et al., 1995). Malate dehydrogenase activity, used as a cytoplasmic marker, was assayed in the direction of oxalacelate reduction (Reng et al., 1993). UGPase and AGPase activity were determined in either the pyrophosphorolysis or the synthesis direction, as described in Sowokinos et al. (1993) and Ballicora et al. (2003). Hexokinase, hexose-P isomerase, and hexose-P mutase were assayed according to Pearce and Carr (1969).

The $K_i$ values for UDP and ADP (the enzyme-inhibitor dissociation constant for product-competitive inhibition) for SPS-A and SPS-B were determined with homogeneous preparations of each isoenzyme (Porchia and Salerno, 1996; Cumino et al., 2002). Anabaena hexokinase and UGPase kinetic parameters were determined in partially purified enzyme fractions, following a purification procedure similar to that described by Porchia and Salerno (1996).

## Permeabilization of Anabaena Cells and in Situ Suc Synthesis

Filaments of *Anabaena* sp. PCC 7120 grown autotrophically in $BG11_0$ or BG11 medium were permeabilized as previously described (Salerno et al., 2004). The permeabilization of heterocysts was performed according to Bottomley et al. (1980). In situ Suc synthesis was determined in the assay mixture (50 $\mu$L total volume) containing 5 mM UDP-[U-$^{14}$C]Glc or ADP-[U-$^{14}$C]Glc (specific activity $2 \times 10^5$ cpm $\mu mol^{-1}$ or $4.4 \times 10^5$ cpm $\mu mol^{-1}$, respectively), 10 mM Fru-6P, 100 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, and an aliquot of toluene-treated cells. Labeled Suc was separated by paper chromatography and quantified as previously described (Porchia and Salerno, 1996).

## Carbohydrate and Metabolite Determination

Suc and glycogen were determined in ethanol extracts as described by Geigenberger et al. (1996). Suc content was quantified by measuring Fru and Glc after hydrolysis with acid invertase using a coupled-enzyme method (Puebla et al., 1997). For glycogen determination, pellets of the ethanol extraction were solubilized by heating to 95°C in 0.1 N NaOH for 30 min. After acidification to pH 4.9 with an HCl/sodium-acetate mixture (pH 4.9), part of the suspension was digested overnight with amyloglucosidase and amylase. The intermediate metabolites, Fru-6P, Glc-6P, Glc-1P, UDP-Glc, and Suc-6P, were determined in perchloric acid extracts using an enzymatic cycling assay (Salerno et al., 1996). Enzymes and chemicals for biochemical analyses were obtained from Boehringer and Sigma-Aldrich.

## Metabolic System Delineation

Control of the steady-state behavior of the Suc-glycogen metabolism relationship in Anabaena $N_2$-fixing filaments was determined through metabolic control analysis, as developed by Kacser and Burns (1973) and Heinrich and Rapoport (1974). The capabilities of this mathematical model are not limited to enzymes obeying the Michaelis-Menten formulation, but can also be extended to complex rate expressions. The enzymatic reactions simulated in this model respond to kinetic mechanisms different from those described in a previous maize (*Zea mays*) leaf model (Barreiro, 1999). This method also allows introduction of flux inhibition and activation mechanisms into the model.

To restrict the model to reactions directly involved in Suc synthesis and degradation, we omitted the Calvin cycle and pentose-P pathway, the major pathway of cyanobacteria Glc catabolism (Summers et al., 1995). Elementary flux modes were calculated using METATOOL software (Pfeiffer et al., 1999). Steady-state calculation of the kinetic models was performed using Gepasi version 3.30 (Mendes, 1997).

The kinetic parameters and thermodynamic data for all enzymes used in the model are summarized in Table I. Kinetic constant values were either taken from the literature, where available, or estimated, as indicated in Table I. The reactions in this model were assumed reversible unless information on irreversibility in vivo had been reported (Rohwer and Botha, 2001). The fixed extracellular metabolite concentrations were 10 mM Fru, 10 mM Glc, 5 mM ATP, 5 mM UTP, and 2 mM 3-phosphoglycerate. The model was validated and its performance assessed against independent experimental observations of the determination of final products (Suc and glycogen) and intermediate metabolites (Fru-6P, Glc-6P, Glc-1P, UDP-Glc, and Suc-6P). Maximal enzyme activity, metabolite concentration, and flux value were determined. For metabolic flux measurements, *Anabaena* sp. PCC 7120 cells were grown in $BG11_0$ medium and harvested by centrifugation in the midlight phase. Data were obtained from independent experiments conducted in triplicate. Cells were disrupted by sonication and incubated with extracellular fixed metabolites. Metabolites and enzyme activities were measured in aliquots of the same sample taken at six different time points.

## Isolation, Manipulation, and Analysis of Nucleic Acids

Plasmids were isolated and modified according to standard protocols (Sambrook and Russell, 2001). Genomic DNA was isolated from cyanobacteria as previously described (Cai and Wolk, 1990). RNA from Anabaena was obtained according to Vargas et al. (2003). RNA quality and PCR products were analyzed by electrophoresis in 1% agarose gels.

## RT-PCR

For RT-PCR analysis, total RNA treated with DNase (RQ1 RNase-free DNase; Promega) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and specific primers for *spsA*, *spsB*, and *sppA* (Cumino et al., 2001, 2002). The primers designed for AGPase gene detection were *agp*-f (5′-CGGGATCCATGAAAAAAGTCTTAGCAATTATTCT-3′) and *agp*-r (5′-CGGAATTCGCTAAATGATTGTGCCATCTGTAATAAC-3′). PCR reactions were run on a PTC-100 thermal cycler (model-96V; MJ Research)

for 25 cycles of 94°C (1 min), 50°C (1 min), and 72°C (1 min) plus a single step at 72°C for 5 min. To determine the optimal amount of input RNA, the 3-fold diluted template RNA was amplified in RT-PCR assays under identical reaction conditions to construct a standard curve for each gene product. Once the optimal amount of input RNA was determined for each gene product, RT-PCR was carried out under identical reaction conditions to detect differential transcript levels of genes in the distinct N source cultures. Under these conditions, PCR amplification occurs in the linear range. Amplification of the encoding gene of the subunit of ribonuclease P (*rpnB*) was used as an internal control in RT-PCR determinations, as described by Zhu et al. (2001). Control reactions were incubated at 30°C for 30 min in the presence of RNaseA to ascertain RNA dependence on the RT-PCR signals. Control reactions were incubated at 30°C for 30 min in the presence of RNaseA to ascertain RNA dependence on the RT-PCR signals. Blots were quantified using an imaging analyzer (Fotodyne model express zoom lens system) and its dedicated software (TotalLab image analysis software).

## Construction of GFP Fusion Reporters

To construct the *spsA* promoter::*gfp* ($P_{spsA}$::*gfp*), *spsB* promoter::*gfp* ($P_{spsB}$::*gfp*), *sppA* promoter::*gfp* ($P_{sppA}$::*gfp*), and *nifHDK* promoter::*gfp* ($P_{nifHDK}$::*gfp*) transcriptional reporters, three DNA fragments containing upstream sequences of the translational start codons of *spsA*, *spsB*, *sppA*, and *nifHDK* were amplified from *Anabaena* sp. PCC 7120 genomic DNA by PCR using the primers *spsA*-f (5′-GTCTTTTGATATATACTTTCTGCATAGCTT-3′) and *spsA*-r (5′-ACCACCGGCTTCTTCTTGACCTATTTC-3′); *spsB*-f (5′-GCTATAGTTGTGATTTCTTACGTATAT-3′) and *spsB*-r (5′-TGCCCTCCAGCTTCTTCTTTGCCAATTT-3′); *sppA*-f (5′-CCGTCGGGAATCTGAAATAAAGCGTATA-3′) and *sppA*-r (5′-TACTCTGGTAGGTAATGATGCAGCCCTGGC-3′); and *nif*-f (5′-CTGAGACTGCACATCAAGGTAGAAG-3′) and *nif*-r (5′-TGTTCTCTTTTCCTGCAATTGGTTG-3′).

Amplification products were of 950, 477, 559, and 1,000 bp, respectively. Each DNA fragment was ligated to pGEM-T Easy (Promega). After digestion with *Eco*RI, each resulting DNA fragment was ligated into a shuttle vector derived from pAM505 and pKEN2-GFPmut2 (source of the *gfp*-mut2 sequence) in frame with the GFP coding sequence (Yoon and Golden, 2001). The resulting conjugal plasmids, pAM154, pAM287, pAM342, and pAM1000, containing *spsA-gfp*, *spsB-gfp*, *sppA-gfp*, and *nifHDK-gfp* transcriptional fusions, respectively, were confirmed by restriction digestions and DNA sequencing. Plasmid pAM1000 was used as a control for localization of GFP in heterocysts and plasmid pAMC486, containing the large subunit of Rubisco gene promoter ($P_{rbcL}$), was used to detect GFP expression in vegetative cells (Yoon and Golden, 2001; Curatti et al., 2006). Strains carrying the promoterless *gfp* construct pAMpmt$^-$ were used as negative controls. Plasmids were transferred by conjugation into *Anabaena* sp. PCC 7120 based on the method described by Elhai and Wolk (1988). Plasmids pAM505, pKEN2-GFPmut2, and pAMC486 were kindly provided by Dr. James W. Golden (Texas A&M University).

## Fluorescence Analysis and Microscopy

Cells grown in $BG11_0$ were used for photography. Fluorescence and phase contrast micrographs were taken on a Nikon microscope (model E600) with an X100 objective using specific emission filter sets. GFP fluorescence images were taken by illumination with light (450- to 490-nm wavelength) and photographing emission through a filter of 510-nm wavelength narrow band pass with a 3-s exposure. Red emission from photosynthetic pigments was photographed without the 510-nm filter. Images were captured with a Nikon digital camera (model E995) attached to the microscope and processed with Adobe Photoshop version 4.0 software. The presence of a thick cell envelope, changes in cytoplasm granularity, cyanophycin granulate formation at the cell poles, and absence of chlorophyll fluorescence distinguished heterocysts from vegetative cells (Elhai and Wolk, 1990).

## Primer Extension Mapping of tsps

Primer extension experiments were performed according to Sambrook and Russell (2001) from total RNA (30 $\mu$g) and using SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen) and oligonucleotides B-*ol*-tsp3 (5′-TTTGTTCAGCATTTACATTTAATCTCTTTA-3′), B-*ol*-tsp4 (5′-TGCCCTCCAGCTTCTTCTTTGCCAATTT-3′), covering positions −185 to −155 and +61 to +89, with respect to the *spsB* translational start, respectively. Primers were end labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Invitrogen). cDNA products were purified and resolved on a sequencing gel along sequencing ladders generated using the same primers as those used in the primer extension experiments. Nucleotide sequencing was carried out using the dideoxy-chain termination method with a Sequenase quick-denature plasmid sequencing kit (USB Corporation).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ302071 and AJ302073.

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