Inorganic Carbon Limitation Induces Transcripts Encoding Components of the CO₂-Concentrating Mechanism in \textit{Synechococcus} sp. PCC7942 through a Redox-Independent Pathway

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The cyanobacterial CO₂-concentrating mechanism (CCM) allows photosynthesis to proceed in CO₂-limited aquatic environments, and its activity is modulated in response to inorganic carbon (Ci) availability. Real-time reverse transcriptase-PCR analysis was used to examine the transcriptional regulation of more than 30 CCM-related genes in \textit{Synechococcus} sp. strain PCC7942 with an emphasis on genes encoding high-affinity Ci transporters and carboxysome-associated proteins. This approach was also used to test hypotheses about sensing of Ci limitation in cyanobacteria. The transcriptional response of \textit{Synechococcus} sp. to severe Ci limitation occurs rapidly, being maximal within 30 to 60 min, and three distinct temporal responses were detected: (a) a rapid, transient induction for genes encoding carboxysome-associated proteins (\textit{ccmKLMNO}, \textit{rbcLS}, and \textit{icfA}) and the transcriptional regulator, \textit{cmpR}; (b) a slow sustained induction of \textit{psbAII}; and (c) a rapid sustained induction of genes encoding the inducible Ci transporters \textit{cmpABCD}, \textit{sbtA}, and \textit{ndhF3-D3-chpY}. The Ci-responsive transcripts investigated had half-lives of 15 min or less and were equally stable at high and low Ci. Through the use of a range of physiological conditions (light and Ci levels) and inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea, glycolaldehyde, dithiothreitol, and ethoxyzolamide, we found that no strict correlation exists between expression of genes known to be induced under redox stress, such as \textit{psbAII}, and the expression of the Ci-responsive CCM genes. We argue that redox stress, such as which occurs under high-light stress, is unlikely to be a primary signal for sensing of Ci limitation in cyanobacteria. We discuss the data in relation to current theories of CO₂ sensing in cyanobacteria.
transcriptional regulators of the ndhF3/D3/chpY (cupA) and cmp operons, respectively (Figge et al., 2001; Omata et al., 2001), whereas RbcR is thought to be involved in control of the rbc operon (Mori et al., 2002). Despite the rapid progress in defining the genetic basis of the cyanobacterial CCM, the first integrated study of the transcriptional response of CCM-related genes to Ci limitation was published only recently, from work in *Synechocystis* (McGinn et al., 2003). One of the key findings of this study was that the expression of inducible, CCM-related genes is modulated in response to changes in external Ci in as little as 15 min.

The signaling pathways that lead to up-regulation of the CCM in response to Ci limitation remain to be defined. It is unclear what sort of signal is actually perceived by cells subject to this stress. A number of competing theories have been proposed (for review, see Kaplan and Reinhold, 1999). These include direct sensing of external Ci or the internal Ci pool, the detection of changes in the concentration of photorespiratory or Calvin cycle intermediates, or changes in the redox potential of the photosynthetic electron transport chain. In support of the latter theory, a link between light stress and the low-Ci response of the CCM has recently been suggested with reports that some CCM-related genes are light responsive in *Synechocystis* (Hihara et al., 2001; Huang et al., 2002). However, this finding was not replicated in another recent study (McGinn et al., 2003).

The nature of the low-Ci signal remains elusive, and an integrated picture of the transcriptional regulation and physiological expression of the CCM is not yet available in *Synechococcus*, which has been favored for physiological and genetic studies of the CCM (Price et al., 2002). Accordingly, this study had two goals. The first goal was to describe aspects of the transcriptional response of *Synechococcus* cells to severe and intermediate Ci limitation using highly sensitive real-time PCR assays and to place these observations in a physiological context. The second goal of this study was to examine some competing theories about the nature of the low-Ci signal that elicits induction of a high-affinity CCM. We detected distinct temporal responses to Ci limitation at the transcriptional level, and our results suggest it is unlikely that *Synechococcus* cells sense Ci limitation through a redox-sensing mechanism such as that which underpins the high-light response. Instead, we propose that depletion of the internal Ci pool or changes in phororespiratory activity may be the primary signal that is sensed by a Ci-limited cell.

RESULTS

Early Transcriptional Response of *Synechococcus* to Severe Ci Limitation

The responsiveness of several potential housekeeping genes to inorganic Ci limitation, including *gap1* and *ppc*, encoding glyceraldehyde-3-phosphate dehydrogenase and phosphoenolpyruvate carboxylase, respectively, was determined. Exponentially growing *Synechococcus* high-Ci cells (bubbled with 1.7% CO₂ in air) were harvested and immediately transferred to CO₂-free air equilibrated buffer and aerated with CO₂-free air for 3 h. First-strand cDNA was generated from normalized total RNA extracts from treated cells and quantitative real-time reverse transcriptase (RT)-PCR assays, using SYBR Green I to monitor product formation, were performed using *ppc*-specific primers (Table I). The *gap1* transcript was found to vary considerably (results not shown), but the abundance of *ppc* was found to be relatively unresponsive to Ci limitation, varying by less than 35% in two independent experiments (Fig. 1). Accordingly, *ppc* was used to normalize for small variations in starting template concentration and the efficiency of cDNA synthesis in all further experiments. To provide a “snapshot” of the early transcriptional response in *Synechococcus*, cells were subjected to a severe Ci limitation (as above) for 30 min. As a control, a culture was harvested, resuspended in high-Ci equilibrated medium, and returned to aerated with 1.7% CO₂. The abundance of 35 transcripts encoding membrane transporters, carboxysome-associated genes, electron transport components, and regulatory factors in low-Ci-induced cells relative to...
high-Ci-grown cells was determined by real-time PCR (Fig. 2) using the gene-specific primers listed in Table I.

As has been observed in Synechocystis, we found that expression of transcripts encoding the known inducible Ci transport activities was induced by Ci limitation in Synechococcus. Previously, only expression of the cmp operon had been confirmed as being low-Ci responsive in this strain (Omata et al., 1999, 2001). Genes contributing to the high-affinity CO₂ transporter NDH-I_3, ndhF3-ndhD3-chpY (cupA) are cotranscribed in Synechocystis, and expression of members of this operon has been reported to be induced by 100% to 200% upon Ci limitation (Ohkawa et al., 1998; McGinn et al., 2003). By contrast, we found the degree of induction of these transcripts to be an order of magnitude higher in Synechococcus. The low-Ci induction of the sftA and cmpA-D transcripts, encoding the two inducible, high-affinity HCO₃⁻ transporters was also orders of magnitude higher in Synechococcus than has been reported for Synechocystis (McGinn et al., 2003). This difference may reflect the fact that real-time PCR assays have a much greater dynamic range than other methods of gene expression analysis such as semiquantitative RT-PCR and microarrays, which tend to underestimate large changes (Schmittgen et al., 2000). Like Synechocystis, the genes encoding the low-affinity, constitutive CO₂-transport activity, ndhF4/D4 and chpX, were found to be constitutively expressed. We also assayed the Ci-responsive expression of three other putative membrane transporters. It has previously been proposed that dc14 might encode a HCO₃⁻ transport activity in Synechococcus (Bonfil et al., 1998), whereas cynA, the substrate binding protein from an ABC-transporter of the mono-anion group (Maeda et al., 2000), although ascribed a function in CN⁻ transport, could potentially bind HCO₃⁻. Also, expression of a gene encoding a putative membrane transporter (termed pmt in this study) has been suggested to be low-Ci responsive in Synechococcus (O.A. Koksharova, submission notes for GenBank accession no. AF428100). Our results suggest that the expression of all of these three genes is unresponsive to Ci limitation.

Carboxysomes are known to increase in number in Synechococcus cells in response to Ci limitation (McKay et al., 1993), and corresponding increases in Rubisco and carboxysomal-CA activity (Price et al., 1992) and rbcL/S and ccmKLMNO transcript abundance have been reported (Omata et al., 2001). Compared with transcripts encoding the inducible transporters, we detected relatively small increases (approximately 200%–500%) in the abundance of rbcL and ccmKLMNO in response to Ci limitation and detected for the first time, to our knowledge, induction of the icfa transcript, which encodes carboxysomal CA. We could not detect expression of the open reading frame ccmI (result not shown) under either Ci

Figure 2. Early transcriptional response of Synechococcus cells subject to a severe Ci limitation. Exponentially growing high-Ci cells were swapped to CO₂-free air equilibrated buffer and bubbled with CO₂-free air for 30 min. The relative abundance of specific transcripts encoding known or putative Ci transporters (A), carboxysome associated proteins (B), or electron transport or regulatory proteins (C), was determined by real-time PCR. Bars represent the extent of induction or repression after the shift to low Ci as a percentage of the high-Ci amount (set at 100%). SE for three separate measurements. Similar data were obtained from a replicate experiment (data not shown). Note the break in the y axis in A and C. The scale is exponential after the break in A.
regime. Some studies have found no induction of carboxysome-associated genes (McGinn et al., 2003) after 60 min in *Synechocystis*, whereas one study detected a small increase (60%–70%) in rbcL transcript abundance in cells subject to 90-min Ci limitation (Omata et al., 2001). These results may reflect a genuine difference between the strains given a recent finding that Ci limitation is not accompanied by an increase in carboxysome number in *Synechocystis* cells (So et al., 2002).

Severe Ci limitation is somewhat analogous to a high-light stress because depression of CO₂ fixation would cause over-reduction of the plastoquinone pool. A preliminary report of the low-Ci inducibility of psbAII has emerged from another group (T. Omata, personal communication). We also found low-Ci-responsive expression of transcripts encoding other components of the photosynthetic electron transport chain. The psbAII/III and psbDII/II gene families, encoding alternative D1 and D2 proteins, responded to Ci limitation as they do to high-light stress (Schaefer and Golden, 1989; Bustos and Golden, 1991). That is, we found that psbAII expression was down-regulated in response to Ci limitation, whereas psbAII/III and psbDII expression was up-regulated. Other genes such as glnB, encoding the PII-signaling protein, and dc13, encoding a putative methyltransferase, have been proposed to have a potential regulatory role in low-Ci sensing (Ruppert et al., 2002; Amoroso et al., 2003), however, neither was transcriptionally regulated by a 30-min Ci limitation.

As with other Lys-R-type transcriptional regulators, expression of ndhR, a controller of the ndhD3/F3/chpY operon, and the cmpA-D operon regulator, cmpR, is stress responsive, and transcript abundance for these genes is induced in response to Ci limitation (Figge et al., 2001; McGinn et al., 2003). Although cmpR has been identified in *Synechococcus* (Omata et al., 2001), an ndhR homolog has not yet been identified in this strain. We also found that cmpR transcript is more abundant in Ci-limited cells in this strain.

### Table 1

Sequences of the gene-specific primers used in real-time PCR assays of gene expression in *Synechococcus* sp. PCC7942

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a Putative membrane transporter.  b See “Materials and Methods.”

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believed to encode a regulator of the rbcLS operon by virtue of the function of its proteobacterial homolog (Mori et al., 2002), was not transcriptionally induced by Ci limitation in this study.

Temporal Response of CCM-Related Gene Expression in *Synechococcus* Cells Subject to Severe Ci Limitation

To date, there has been no report of the kinetics of low-Ci-inducible gene expression using quantitative gene expression assays. High-Ci-grown *Synechococcus* cells were transferred to low Ci as described above, and cultures were sampled at 15, 30, 60, and 180 min after the switch. Changes in the abundance of a representative subset of transcripts from the full set previously assayed, compared with time zero, were quantified as before using real-time PCR assays. The low-Ci-inducible transcripts exhibited kinetic patterns of three types. First, the abundance of one transcript, psbAII, increased steadily over the first 60 min and was sustained at this level for 3 h (Fig. 3A). A second group of transcripts, encoding carboxysome-associated proteins and cmpR, was rapidly induced but, in the case of ccmM, rbcL, and icfA, returned to uninduced levels approximately 60 min after induction and in the case of cmpR, by 3 h (Fig. 3B). It is possible that carboxysome gene expression may also be strongly but transiently Ci responsive in *Synechocystis* and that there is no significant difference in the regulation of these genes between the two strains. A third group of transcripts, encoding the inducible Ci transport activities, was rapidly induced to maximum amounts by 30 to 60 min (Fig. 3C) and was maintained at relatively high amounts over the 3-h time course. A slight transient reduction in the amount of rbcR transcript was detected at 15 min, whereas the amount of transcript for psaE slowly declined over the 3 h (Fig. 3A).

The physiological response of *Synechococcus* cells to Ci limitation was also monitored for 3 h after the transition from bubbling with 1.7% CO₂ to bubbling with CO₂-free air. The maximum photosynthetic rate of cells during this period was largely constant, indicating that cells were not photo-inhibited (Fig. 3D). The relative affinity for Ci, as determined by net $K_{0.5} (Ci)$, started to increase soon after transfer to Ci limitation (Fig. 3D). The maximal increase in affinity was achieved between 1 and 1.5 h (Fig. 3D), similar to what has been reported recently in *Synechocystis* (McGinn et al., 2003) under comparable conditions. This response is faster than previously observed for *Synechococcus* (Yu et al., 1994) and is largely due to a more rapid transfer between Ci conditions. The initial increase in affinity for Ci at 30 and 60 min was abolished by treating cells with 200 μg mL⁻¹ protein synthesis inhibitor chloramphenicol (results not shown). The initial increase in affinity, therefore, was due to a genuine increase in de novo protein synthe-

![Figure 3](https://www.plantphysiol.org/content/dam/plantphysiol/bre/PlantPhysiol/v133/i1/a316-fig3.png)

**Figure 3.** Time course of CCM-related transcript expression in severely Ci-limited *Synechococcus* cells and induction of a high-affinity CCM. A through C, Relative abundance of a subset of CCM-related transcripts as determined by real-time PCR. Symbols represent the extent of induction or repression after the shift to low Ci at each time point as a percentage of the high-Ci amount (set at 100%) ± se for three separate measurements. D, Maximum photosynthetic rate ($V_{max}$) and $K_{0.5}(Ci)$ for cells collected at each time point. Similar data were obtained from a replicate experiment (data not shown). Note the break in the y axis in part C. The scale is exponential after the break in C.
sis and is unrelated to the fast induction response in *Synechococcus* that appears to result from allosteric activation of an existing HCO$_3^-$ transporter (Sültemeyer et al., 1998).

**Relaxation of the High-Affinity CCM in *Synechococcus* Cells**

Relatively little data exists about the relaxation of high-affinity CCMs and consequently the rate of turnover of CCM components. To address this question, exponentially growing high-Ci *Synechococcus* cells were transferred to CO$_2$-free equilibrated growth medium and bubbled for 2 h with CO$_2$-free air. Cells were then swapped to bubbling with 1.7% CO$_2$ (designated time zero) and supplemented with NaHCO$_3$ to a final concentration of 5 mM. This amount of supplementary Ci equals the total concentration of Ci found in actively growing cultures bubbled with 1.7% CO$_2$ under steady-state conditions (data not shown). Cultures were sampled over the next 48 h to determine the relative affinity for Ci, as $K_{0.5}$ (Ci) (Fig. 4). The $K_{0.5}$ (Ci) decreased from 163 to 15 µM during the initial CO$_2$-free air induction. For 25 h after the return to high-CO$_2$ conditions, the $K_{0.5}$ (Ci) rose steadily to 133 µM, about 80% of the initial high-CO$_2$ value, but cells required a further 24 h growth at high CO$_2$ to return to the initial low-affinity state. Previous work on the relaxation of the high-affinity CCM in *Synechocystis* found that it took considerably longer (over 40 h) for cells to approach a low-affinity state (Benschop et al., 2003), suggesting that a difference exists in regulation of this response between the two strains.

To examine the turnover and stability of CCM-related transcripts in cells subject to either Ci limitation or Ci sufficiency, an exponentially growing high-Ci *Synechococcus* cell culture was transferred to CO$_2$-free equilibrated growth medium and bubbled for 30 min with CO$_2$-free air. This period is sufficient to strongly induce Ci-responsive CCM transcripts but brief enough to capture the transient increases observed for transcripts such as *ccmM* and *cmpR* (Fig. 3, A–C). Subsequently, the culture was split four ways. Two cultures were supplemented with 5 mM NaHCO$_3$ (designated time zero) and swapped to bubbling with 1.7% CO$_2$ in the presence or absence of 200 µg mL$^{-1}$ of the transcriptional inhibitor rifampicin for a further 2 h. The remaining two cultures were supplemented with 5 mM NaCl to balance sodium, and bubbled with CO$_2$-free air with or without rifampicin as above. The four cultures were sampled for transcript analysis at various times after the treatments. Changes in the abundance of *ccmA*, *sbtA*, *chpY*, *cmpR*, *ccmM*, and *psbAII* compared with time zero, were quantified as before using real-time PCR assays (Fig. 5). The *ccmA* transcript had a half-life of approximately 15 min at both high and low Ci. However, it was even more rapidly degraded in high-Ci cells in the absence of rifampicin, being two-thirds less abundant at 15 min than in high-Ci cells treated with rifampicin. This result suggests that the synthesis of a factor is required for rapid degradation of the *ccmA* transcript. The *sbtA* and *chpY* transcripts responded similarly to *ccmA* (results not shown), however, both *sbtA* and *chpY* exhibited a transient insensitivity to rifampicin treatment followed by rapid degradation. This type of response has been observed in *Escherichia coli* and is thought possibly to result from different sigma factors influencing the interaction of rifampicin with the RNA polymerase holoenzyme (Selinger et al., 2003). The *cmpR* transcript had a half-time of less than 5 min at high and low Ci and was equally rapidly degraded at high Ci in the absence of rifampicin. The *ccmM* transcript was initially insensitive to any of the treatments, but by 15 min, approximately 50% and 75% of the initial transcript pool had decayed at high and low Ci, respectively. The trajectory apparent for *ccmM* abundance in the absence of rifampicin indicates that active transcription still occurred after 2 h at high Ci. The *psbAII* transcript had a half-life of approximately 30 min under both Ci conditions, which is similar to previously reported values for this transcript in cells grown with Ci sufficiency at low or high light (Kulkarni et al., 1992). At high Ci, *psbAII* was slightly more rapidly degraded in the absence of rifampicin. In summary, the CCM-related transcripts investigated had relatively short half-lives (5–15 min), were in general equally stable at high and low Ci, and with the exception of *ccmM*, were not actively transcribed.
at high Ci. This indicates that changes in transcript abundance in response to variations in external Ci availability result from transcriptional control of these genes rather than changes in mRNA stability.

**Effect of Light on CCM-Related Gene Expression**

To assess whether high-light stress is sufficient to induce CCM-related gene expression, as has been reported in *Synechocystis* (Hihara et al., 2001; Huang et al., 2002), a *Synechococcus* culture bubbled with 1.7% CO₂ and illuminated with 85 μmol photons m⁻² s⁻¹ was grown to exponential phase and harvested. An equal number of cells were resuspended in media equilibrated with 1.7% CO₂ or CO₂-free air and then bubbled with 1.7% CO₂ or CO₂-free air, respectively. Duplicate cultures were illuminated with 85 or 500 μmol photons m⁻² s⁻¹ (termed low or high light) for 30 min. At high Ci, high-light treatment was not found to be sufficient to induce increased expression for any of the CCM-related genes tested, whereas *psbAII* transcript abundance increased around 2.10³% (Fig. 6). At low Ci, a high-light treatment increased the degree of induction around 4-fold for the *cmpA* transcript and by about 50% for *psbAII* compared with that observed in cells subject to a low-Ci/low-light treatment (Fig. 6). The abundance of *sbtA*...
and chpY transcripts was unresponsive to light under either Ci regime.

**Effect of Redox Modifiers and Inhibitors of CO₂ Fixation or CO₂ Transport on CCM-Related Gene Expression**

To examine some competing theories about the nature of the low-Ci signal that is sensed by *Synechococcus* cells, high-Ci-grown cells were treated for 30 min with 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or 20 μM 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB)—agents that increase the reduction state of the electron carriers between photosystems II and I. Cells were also treated with 5 mM dithiothreitol (DTTred), 200 μM CA of the inhibitor ethoxyzolamide (EZ), and 10 mM of the carbon fixation inhibitor glycolaldehyde (GLY). The concentration of GLY used has only a minor effect on CO₂ transport (Salon et al., 1998). Changes in cmpA, sbtA, and psbAII transcript abundance relative to untreated control cells were determined using real-time PCR assays (Fig. 7A). As previously reported in *Synechocystis*, psbAII transcript abundance increased substantially in cells treated with DTTred and DCMU (Sippolo and Aro, 1999; Li and Sherman, 2000). GLY treatment, which would be expected to emulate a high-light stress due to over-reduction of the photosynthetic electron transport chain, also resulted in an increase in psbAII abundance. These treatments did not alter cmpA or sbtA transcript abundance, with the exception of the GLY treatment, which elicited a small increase in sbtA transcript abundance. However, this increase was 3 orders of magnitude less than that typically observed in low-Ci cells (Fig. 2A). The EZ treatment resulted in nearly a 10-fold increase in the abundance of cmpA and sbtA transcripts, however, this is less than 1% of the response typically observed in low-Ci cells. It has previously been shown that the effect of EZ treatment can be overcome by increasing the availability of Ci (Price and Badger, 1989b). Accordingly, to increase the ratio of EZ to CO₂ within the culture medium, *Synechococcus* cells were instead grown with air delivered through a fritted sparge (approximately 0.4 L min⁻¹). This rapid bubbling technique does not induce a high-affinity CCM (Yu et al., 1994), and compared with high-Ci cells, transcripts such as cmpA were induced to a degree that was less than 1% of that typically observed in severely Ci-limited cells (data not shown). By contrast, with the exception of the rbcL transcript, inducible CCM-related transcripts and psbAII increased significantly in abundance in rapidly air-bubbled cells treated with EZ (Fig. 7B), to a degree comparable with that typically seen in cells subject to a severe Ci limitation (Fig. 2).

We also tested the effect of GLY on low-Ci induction of CCM-related gene expression. Equal volumes of cells from an exponentially growing high-Ci *Synechococcus*
*Synechococcus* culture were harvested and resuspended in medium containing approximately 100 μM total Ci. Cells were then bubbled slowly with air, using a standard pipette (approximately 0.15 L min⁻¹) for 30 min to create a moderate level of Ci limitation. At the swap over, one of the cultures was treated with 10 mM GLY, and cells were incubated in the dark for 5 min. As a control, an equivalent number of high-Ci cells were resuspended in high-Ci-equilibrated medium and returned to bubbling with 1.7% CO₂. Treatment of cells with GLY significantly reduced the low-Ci induction of *chpY* and *cmpR* by more than 80% and that of *cmpA* and *sbtA* by more than 99% (Fig. 7C). By contrast, GLY treatment resulted in a 10-fold greater induction in *psbAII* expression than that observed in the untreated air-bubbled culture.

**DISCUSSION**

We have profiled changes in the expression of genes involved in the CCM of *Synechococcus* when cells are transferred from Ci sufficiency to conditions of Ci limitation. We have also tested a number of ideas about the sensory mechanisms leading to induction of a high-affinity CCM. As has been observed in *Synechocystis*, the transcriptional response of this strain to severe Ci limitation occurs rapidly, reaching a maximum within 30 to 60 min (Fig. 3). One of our key findings includes the characterization of three distinct temporal responses of low-Ci-inducible transcripts. The temporal responses of these transcripts to Ci limitation align with functional groupings of the encoded proteins: (a) a slow, sustained induction for *psbAII*, (b) a rapid, transient induction for genes encoding the carboxysome-associated proteins (*ccmKLMNO* and *rbcL*) and *cmpR*, and (c) a rapid, sustained induction for genes encoding the inducible Ci uptake systems (*sbtA*, *cmpABCD*, and *ndhF3-D3-chpY*). In all cases, the transcriptional response of low-Ci-inducible CCM-related genes preceded the full physiological response by 30 to 60 min, however, the three distinct temporal responses are suggestive of the existence of alternative controls on expression of subsets of these genes. Furthermore, the pattern of decay of CCM-related transcripts at high- and low-Ci suggests that the abundance of these transcripts is primarily controlled at the level of transcription rather than at the level of mRNA stability (Fig. 5).

Previous work has shown that light is a prerequisite condition for induction of CCM-related gene expression (McGinn et al., 2003), and under low-Ci conditions, high light tends to accentuate induction of some CCM-related genes (P. McGinn, unpublished data). However, the nature of the primary signal that elicits induction of a high-affinity CCM is unknown. Direct sensing of external or internal Ci availability, changes in the redox potential of the photosynthetic electron transport chain or in the amounts of intermediates in the Calvin cycle, and photorespiratory pathways have all been proposed (for review, see Kaplan and Reinhold, 1999). It is well established that light-regulated expression of the *psbA* family occurs through a redox-sensing mechanism in cyanobacterial cells (Sippola and Arro, 1999; Alfonso et al., 2000; Li and Sherman, 2000; van Waasbergen et al., 2002). Given the finding that the expression of members of the *psbA* and *psbD* families is responsive to Ci availability and reports that some CCM-related gene expression is light-responsive (Hihara et al., 2001; Huang et al., 2002), a redox-sensing mechanism modulating CCM-related gene expression seems to be an attractive theory. However, the reported culturing conditions of Huang et al. (2002) involved air-mixing of *Synechocystis* cells at 100 rpm, possibly causing a Ci limitation that would be exacerbated by increasing the light intensity. Hihara et al. (2001) found that a low-to-high-light transition for *Synechocystis* bubbled with 1% CO₂ lead to induction of *ndhF3-D3* transcripts, but this result has not been reproduced in another study (McGinn et al., 2003) or in the present study involving *Synechococcus* (Fig. 6).

We detected multiple conditions under which the regulation of a classic light-inducible gene, *psbAII*, and CCM-related genes is uncoupled in *Synechococcus* cells. First, a high-light treatment at high-Ci levels, although sufficient to induce *psbAII* expression, failed to significantly induce any of the CCM genes tested at high Ci (Fig. 6). The only effect of high light was found at low Ci, where *cmpA* transcript abundance was 4-fold higher in cells exposed to the higher irradiance. Our results indicate that any high-light responsiveness of CCM-related gene expression in *Synechococcus* is confined to conditions under which Ci is limiting, and this has also been confirmed in *Synechocystis* (McGinn et al., 2003; P. McGinn, unpublished data). Second, our inhibitor studies in *Synechococcus* also show that the regulation of *psbAII* and CCM-related transcripts can be uncoupled (Fig. 7). At high-Ci, *psbAII* expression was strongly induced under conditions known to produce redox stress, namely by treatment with DCMU, DTT_red, and GLY, but the expression of *cmpA* and *sbtA* was unaffected. Also, at low Ci, GLY treatment largely abolished the low-Ci induction of *cmpA*, *sbtA*, and *chpY* but enhanced *psbAII* induction. Collectively, our results suggest that it is unlikely that *Synechococcus* cells detect low-Ci availability primarily through a redox-sensing mechanism shared with light stress-signaling pathways. In addition, our data indicate that an increased concentration of reactive oxygen species, which is known to potentiate signaling in cyanobacteria (He and Harder, 2002), is unlikely to signal Ci limitation. This is because light stress in isolation failed to induce CCM genes, and GLY treatment, which would be predicted to increase ROS, actually prevented induction (Fig. 7). The results of our inhibitor studies are also inconsistent with the direct
perception by cells of reduced external Ci concentration, because GLY and EZ treatment uncoupled the relationship between external Ci concentration and the degree of CCM-transcript induction.

The preceding arguments point to just two of the proposed sensory mechanisms, namely that changes within the internal Ci pool are perceived directly and dictate the degree of induction of the CCM in *Synechococcus* or that cells sense altered photorespiratory activity. In support of the latter theory, it is already established that *Anabaena variabilis* cells grown at low O₂ are slow to adapt to Ci limitation (Marcus et al., 1983). The CA inhibitor EZ is known to severely reduce the internal Ci pool size in Ci-limited *Synechococcus* cells due to inhibition of active CO₂-uptake systems (Price and Badger, 1989a). It would also be predicted to increase photorespiratory activity because of the decreased ratio of CO₂ to O₂ inside the cell. In this study, the EZ treatment of *Synechococcus* cells grown with rapid air sparging led to increases in the expression of all inducible CCM-related genes tested, to a degree comparable with that observed in cells subjected to severe Ci limitation (Fig. 7). A relatively small effect of EZ on *cmtpA* and *sbtA* abundance was also observed in cells grown at 1.7% CO₂, even though it would be predicted that CO₂ diffusion should be sufficient under these conditions to maintain pool sizes that saturate Rubisco (Price and Badger, 1989c; Reinhold et al., 1991). It is possible that reduced flux through the NDH-1 complex, in the presence of EZ, might elicit this minor effect. In GLY-treated cells at low Ci, we found that normally low-Ci-inducible transcripts encoding CCM components were only weakly responsive to Ci limitation. When CO₂-fixation is blocked by inhibitors such as GLY, a larger intracellular Ci pool develops (Salon et al., 1996). However, GLY acts to inhibit ribulose 1,5-bisphosphate regeneration (Sicher, 1984), inhibiting photorespiration as well as Ci pool depletion. We are currently exploring Ci pool- and photorespiratory-dependent sensory mechanisms further using available mutants with altered internal Ci pools and wild-type cells in which photorespiration has been manipulated.

**MATERIALS AND METHODS**

**Cyanobacterial Strains and Culture Conditions**

Cells of the cyanobacterium *Synechococcus* sp. strain PCC7942 were cultured in modified BG-11 medium (Price and Badger, 1989a) containing 20 mM HEPES-KOH, pH 8.0, at 28°C with a light intensity of approximately 85 μmol photons m⁻² s⁻¹. Aeration was delivered through a pipette with a 1-mm annulus at a flow rate of approximately 0.15 L min⁻¹ unless otherwise indicated. To rapidly induce a high-affinity CCM, exponentially growing cells (OD₅₇₀ of 0.3–0.6) that had been bubbled with an air-CO₂ mixture containing 1.7% CO₂ were harvested by centrifugation at 4,800g for 6 min and resuspended in an equivalent volume of CO₂-free medium. The CO₂-free medium was generated by bubbling growth medium with air passed through CO₂-absorbing soda lime (path-length approximately 1 m) for at least 24 h. Cultures were returned to the original conditions but aerated with CO₂-free air.

**Isolation of Total RNA and First-Strand cDNA Synthesis**

Isolation of total RNA from harvested cell pellets stored at ~80°C was essentially as previously described (McGinn et al., 2003). First-strand cDNA synthesis from 3 to 5 μg of normalized total RNA using 0.1 μM each of the three gene-specific primers described in Table I was performed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Real-Time RT-PCR Assays**

Primers were designed using Jellyfish software (LABVELOCITY, v1.5) and, with the exception of the *psbA* primers, forward and reverse primers in every pair had melting temperatures that varied by less than 3°C and GC contents not greater than 55%. For specificity, it was necessary to anchor the *psbAII/III* forward primers in the 5’-untranslated region and to relax design constraints slightly. Primers for *sbtA* were designed after sequencing of a fragment of *sbtA* obtained with degenerate primers (GenBank accession no. AY365060). All other sequences were obtained from GenBank. The relative amount of specific cDNA templates between different samples was quantified using real-time PCR. HotStarTag PCR kit components (Qiagen, Hilden, Germany) were used in 20-μL reactions containing 3 mM MgCl₂, 100 μM each of dATP, dTTP, dCTP, and dGTP, 0.5 μM each of forward and reverse primers, 0.5 to 1 unit of HotStarTaq, and cDNA template equivalent to 25 ng of total RNA. For every reaction, an RNA sample without reverse transcriptase was included to control for genomic DNA contamination. Product formation was monitored by the inclusion of SYBR Green I at a final dilution of 1:4,000 (Fisher Biotech, Springfield, NJ). Thermocycling was conducted in a Rotorgene 2000 Thermal Cycler (Corbett Research, Sydney, Australia) for 35 cycles consisting of denaturation for 30 s at 95°C, annealing at 54°C for 30 s, extension at 72°C for 30 s, and fluorescence acquisition at 84°C for 15 s. Cycling was preceded by a 15-min 95°C activation step. Specificity of amplification was confirmed through a melt curve analysis of final PCR products by ramping the rotor temperature from 55°C to 99°C at 0.2°C s⁻¹ with fluorescence acquired after every 1°C increase.

**Calculation of Changes in Transcript Abundance**

The amplification efficiencies of primer pairs used in real-time PCR assays (Table I) varied by less than 10% compared with that of the normalizer *ppc*, which was 1.9. These values were determined by the slope of the curve generated by amplification of serially diluted cDNA over at least 3 orders of magnitude (r ≥ 0.965). Accordingly, all primer pairs were nominated as having an efficiency of 2 and -fold changes in transcript abundance were calculated using the 2⁻ΔΔCt method (Livak and Schmittgen, 2001), where ΔCt expresses the difference in Ct between a target sample and the Ct of the sample from the basal condition. The value ΔΔCt expresses the difference between the ΔCt of the normalizer (*ppc*) and the target. -Fold changes were converted to percent change whereby a value of 100% equals no change. All reactions were carried out in triplicate, and the error was propagated using standard methods. The final error for any normalized -fold change incorporates the error for both the basal condition and the normalizer and is asymmetrical (for discussion, see Livak and Schmittgen, 2001).

**Mass Spectrometric Measurements**

Cells were prepared and analyzed in the mass spectrometer as previously described (Sültemeyer et al., 1995; McGinn et al., 2003). Cells were assayed at a chlorophyll density of 2 μg mL⁻¹ in BG11 medium buffered with 50 mM BisTrisPropane-HCl (pH 7.9) and where NaNO₃ had been replaced with 20 mM NaCl. Assays were performed in a thermostatted (30°C) mass spectrometer cuvette allowing membrane inlet analysis of O₂ (mass 32) and CO₂ (mass 44). In the presence of 25 μg mL⁻¹ bovine CA, the CO₂ signal was used as a surrogate measure [Ci] in real time. The maximum rate of net O₂ evolution (Vₘₐₓ) was measured in the presence of 3 mM NaHCO₃ and the photosynthetic affinity for Ci was determined as Kₐₘₐₓ (Ci) that is, the Ci concentration required to reach half the maximum rate of net O₂ evolution. Measurements at low levels of Ci were initiated at around 25 μM O₂ and allowed to progressively increase throughout the Ci range. The light inten-

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sitivity was used was 950 μmol photons m⁻² s⁻¹. The concentration of total C in culture was determined as previously described (Badger et al., 1994).

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LITERATURE CITED


Price GD, Badger MR (1989c) Isolation and characterization of high CO₂-requiring-mutants of the cyanobacterium Synechocystis PCC7942: two phenotypes that accumulate inorganic carbon but are apparently unable to generate CO₂ within the carboxysome. Plant Physiol 91: 514–525


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