Glucose-induced Expression of Carotenoid Biosynthesis Genes in the Dark Is Mediated by Cytosolic pH in the Cyanobacterium Synechocystis sp. PCC 6803*

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The expression of carotenoid biosynthesis genes coding for phytoene synthase (crtB), phytoene desaturase (crtP), β-carotene desaturase (crtQ), and β-carotene hydroxylase (crtR) is dependent upon light in the cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis). We have demonstrated that the expression of the above four genes was also elevated in the dark-adapted Synechocystis cells upon glucose treatment as a consequence of transcriptional activation. Treatment with glucose analogs such as l-glucose, 3-O-methylglucose, 2-deoxyglucose, and mannose, or inactivation of glucose uptake and phosphorylation by deletion mutation of glucose transporter (gICP) and glucokinase (gK), respectively, did not induce up-regulation of carotenoid genes. When respiratory electron transport or coupling to oxidative phosphorylation was inhibited, glucose induction was not observed, indicating that respiratory electron transport per se is not critical for the expression of these genes. In agreement with this view, the extent of gene expression showed a saturation curve with increasing acridine yellow fluorescence yield, without having a close correlation with the ATP contents or ATP/ADP ratio. The results indicate that glucose induction of carotenoid gene expressions is mediated by an increase in cytosolic pH rather than either redox or glucose sensing.

Carotenoids in all photosynthetic organisms including cyanobacteria participate in the light-harvesting process and function in the protection of the photosynthetic apparatus against photo-oxidative damage (1). The cyanobacterium Synechocystis sp. PCC 6803 (hereafter called Synechocystis) contains several genes encoding enzymes involved in carotenoid biosynthesis (2): the crtB gene for phytoene synthase, crtP for phytoene desaturase, crtQ for β-carotene desaturase, and crtR for β-carotene hydroxylase. Despite an abundance of information about the carotenoid biosynthesis pathway and the genes involved, very little is known about the regulation of the expression of carotenoid biosynthesis genes (3).

Light seems to play an important role in the expression of carotenoid biosynthesis genes. In higher plants and green algae, photoreceptors like phytochrome (4, 5) and blue light receptors (6) are involved in light signaling. But, in the cyanobacteria, redox-sensing and signaling by photosynthetic electron transport (7, 8) are probably more important than other photosensory systems. This is because the content of carotenoids in Synechococcus sp. PCC 7942 is proportional to the photosynthetic rate (9), and the expression of phytoene synthase (crtB) and phytoene desaturase (crtP) genes in Synechocystis depends upon light intensity (10).

To address the redox control over carotenogenesis genes, we took advantage of Synechocystis that grows at the expense of an exogenously supplied sole carbon source, glucose (11). Synechocystis cells uptake external glucose by means of a glucose transporter (gICP, the gene locus all0771; Ref. 12) and metabolize it further via glycolysis and the oxidative pentose phosphate pathway (13). NAD(P)H produced from glucose metabolism is oxidized by NADP(H) dehydrogenase in the photosynthetic thylakoid membranes; it modulates the redox status of the plastoquinone (PQ)2 pool. Thus, glucose has been commonly used for the observation of membrane redox-controlled processes in cyanobacteria (14–16).

However, glucose effects on the expression of some genes could result from so-called glucose-sensing and signaling or from changes in the cytosolic redox state, ATP/ADP ratio, or cytosolic alkalinization. In addition to its involvement in redox-sensing by means of respiration, glucose has been known to act as a signaling molecule, which is well documented in higher plants, yeast, and human pancreatic cells. In Arabidopsis, for example, at least three different glucose-sensing systems responsible for up- or down-regulation of photosynthesis, carbon metabolism, and pathogenesis-related gene expression are operative: a hexokinase-dependent system, a hexokinase-independent but as yet unidentified glucose sensor or transporter, and a system dependent upon active glycolysis downstream from hexokinase (for reviews, see Refs. 17 and 18). Therefore, the interpretation of glucose induction of gene expression solely...
in terms of redox-sensing should be made cautiously in higher plants. The cyanobacterium *Synechocystis* is not an exception either, because it contains potential components for glucose signaling cascades, such as hexose transporter, glucokinase (the locus *sll0538*), and other signaling components found in the genomic DNA sequence.

In addition to changes in the redox state of respiratory electron transport, glucose treatment also affects the cytosolic redox state, ATP/ADP ratio, and pH in the cytosol, which are important factors of metabolism (19), glucose repression of young genes (17), and hormone signaling (18, 20, 21). Thus, there is a possibility that glucose induction of some of the above-mentioned photosynthesis genes is under the control of the cytosolic redox status (NAD(P)/H/NAD(P)+), ATP/ADP ratio, or cytosolic pH. In this respect, the translation machinery was suggested to be regulated by photosynthetic electron transport via the proton gradient and/or ATP concentration in higher plants (22) and the cyanobacterium *Synechococcus* (23).

While working on light induction of carotenoid genes with respect to redox-sensing in *Synechocystis*, we found that glucose alone in the dark is enough to stimulate the gene expression. To address whether glucose induction arises from glucose-sensing or whether it is the consequence of respiratory electron transport, glucose analogs, and various electron transport−glucose uptake−, and glucokinase-defective mutants. Our results clearly showed that glucose induction of carotenoid synthesis genes is not mediated by either glucose signaling or redox status of respiratory electron transport but is rather mediated by changes in the pH of the cytosol. To our knowledge, this is the first report suggesting that cytosolic pH change is critical for gene regulation in photosynthetic prokaryotic systems.

**EXPERIMENTAL PROCEDURES**

**Cell Strains, Growth Conditions, and Inhibitors—**Cells of *Synechocystis* sp. PCC 6803 wild-type (WT) and various mutants defective in gene expression of *carotenoid biosynthesis* genes were amplified by PCR using primers of *crtP*, *crtB*, and *crtQ* with RNase-free DNase (Promega). The expression levels of both genes showed that expression of both genes can be regulated by light (10). To determine the extent of light regulation of the key carotenoid biosynthesis genes, *crtO*, *crtQ*, and *crtB* were analyzed in addition to *crtB* and *crtP*. Phototrophically grown cells at the exponential growth phase were subjected to dark treatment for 16 h and then exposed to high light (100 μmol m−2 s−1) for 30 min. RT-mediated PCR analysis showed that dark-adapted cells had only small amounts of mRNAs of all four *crt* genes, but 30 min of illumination at high light intensity increased significantly the levels of mRNAs. RT-PCR analysis was carried out by using total RNA and specific primers of *crtB* (forward, 5'-CTTTACGCTTC-CGGTACCA-3' and reverse, 5'-GGTTACATAGGCGACAGGCT-3'), *crtP* (forward, 5'-GGGAGGGAGGAATACATT-3' and reverse, 5'-CACCTGGATTTAAGGACT-3'), *crtQ* (forward, 5'-AGTTGCTTCAGGAAAGGCTT-3'), and *crtR* (forward, 5'-CTTTTGGTCAGTGGCAAT-3' and reverse, 5'-AATTGGGCTTGGTAGTGTA-3'), and *rns16SS* (forward, 5'-CAGCTTGGAG-TGAGACGG-3' and reverse, 5'-CCACGCTGATGCCTCGTC-3'). One microgram of total RNA was used for each reverse transcription in a 20 μl reaction volume, and 0.8 μl of the reaction mixture was subjected to PCR amplification in an MJ Research Minicycler under the following conditions: initial denaturation at 95 °C for 1 min, followed by 25 (crtB, *crtP*, and *crtQ*), 28 (crtB), and 8 (rns16SS) cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s, and then a final extension step at 72 °C for 7 min. These RNA concentrations were within the linear response range of the PCR amplification (data not shown). The 16S rRNA was included as a positive control. To detect possible DNA contamination, control reactions were performed without RT but with Taq polymerase. The PCR products were routinely analyzed on a 1.4% agarose gel and viewed with a GELDOC2000 densitometer and a computer-aided image analysis system (Bio-Rad). The identities of all RT-PCR products were confirmed by DNA sequencing.

**Adenylate Contents—**Synechocystis cell pellets (2 μg of chlorophyll) treated with various electron transport inhibitors or uncoupler with or without 10 mM glucose for 15 min in the dark were resuspended in adenylic extraction media (1 × CCLR, Promega) and then incubated at 65 °C for 10 min. After centrifugation at 12,000 × g for 5 min at 4 °C, the supernatant was used for the measurement of adenylic contents. ATP and ADP contents were measured by luminometry (MicroLUMAT LB960, EG & G Berthold, Bad Wildbad, Germany) using the chemiluminescent ENLITEN™ ATP assay system (Promega). To determine the ADP concentration, ADP was converted to ATP using pyruvate kinase with buffer (30 mM Hepes-NaOH, pH 7.4, 4 mM MgCl2, 0.125 mM phosphoenol pyruvate) for 1 h at 30 °C. Enzyme was inactivated by heating the reaction mixture for 10 min at 95 °C.

**Acridine Yellow Fluorescence—**Fluorescence yield of acridine yellow from dark-adapted intact cells which had been suspended in the culture media buffered with 5 mM TES-NaOH, pH 8.0, was measured using Xe-PAM (Walz, Effeltrich, Germany) at 30 °C. Acridine yellow was shown to be the best pH-sensitive indicator for the energization of *Synechocystis* (27). For the excitation and detection of acridine yellow fluorescence, the emitter and detector was equipped with band-pass filters (365 nm for emission and 480 nm for excitation). The excitation for acridine yellow was 5 μm and that of chlorophyll was 80 μg.

**RESULTS**

**Expression of Carotenoid Biosynthesis Genes in Response to Light and Glucose—**Expression of carotenogenesis genes, *crtB* and *crtP*, is up-regulated upon illumination of dark-adapted *Synechocystis* cells (10). To determine the extent of light regulation of the key carotenoid biosynthesis genes, *crtO*, *crtQ*, and *crtB* were analyzed in addition to *crtB* and *crtP*. Phototrophically grown cells at the exponential growth phase were subjected to dark treatment for 16 h and then exposed to high light (100 μmol m−2 s−1) for 30 min. RT-mediated PCR analysis showed that dark-adapted cells had only small amounts of mRNAs of all four *crt* genes, but 30 min of illumination at high light intensity increased significantly the levels of mRNAs accumulated (Fig. 1). *crtO* mRNAs were not easily detectable even by RT-PCR analysis (data not shown) and hence were not characterized further. The promoter analysis of both *crtB* and *crtP* genes showed that the expression of both genes can be photoactivated (10). If this is the case, it would be expected that expression of *crt* genes should be subject to light signaling by means of either the photosynthetic apparatus or photoreceptor systems. To address whether redox status of electron transport is sufficient to induce gene expression, cells were exposed to glucose under dark conditions to exclude any involvement of photoreceptor systems. Upon addition of glucose to dark-
adapted cells, all gene transcripts investigated were clearly observed even after 15 min of glucose treatment (Fig. 1).

Treatment of cells with rifampicin, a transcription inhibitor, inhibited accumulation of mRNAs of all genes, suggesting that glucose induction is a consequence of transcriptional activation. From these results, it is apparent that accumulation of the four carotenoid biosynthesis genes is induced not only by light but also by glucose in the dark.

Further Glucose Metabolism, Rather than Glucose Phosphorylation by Hexokinases, Is Required for the Glucose Induction of Carotenogenesis Genes—Glucose induction for the expression of carotenoid biosynthesis genes is likely to be related to either glucose sensing, changes in membrane or cytosolic redox status, or changes in cytosolic energy status as a consequence of respiratory electron transport. First, we asked whether glucose-induction of crt genes is a consequence of glucose signaling as in higher plants (17, 18). A glucose transporter (sbr0771, glcP) was insertionally inactivated (∆glcP) to see whether glucose uptake is required for glucose induction of crt genes. To investigate whether hexokinases act as a sensor, in addition to their catalytic activity, we made an insertional mutant of glucokinase (glcP)−defective (∆glcP) wt cells in both cytochrome c oxidase-defective and quinol oxidase-defective strains (data not shown).

Loss of glucose induction of four crt genes was also observed in WT cells treated with respiratory electron-transfer inhibitors, KCN (inhibitor of terminal oxidases, CtaI and Cyd) or CCCP (an uncoupler), but not with DBMIB (inhibitor of electron flow to CtaI, not Cyd), which only partially inhibited glucose induction (Fig. 3). This partial inhibition was also observed when Cyd-defective cells were treated with DBMIB together with glucose (data not shown). These data imply that glucose induction of the four crt genes is mediated by electron flow through the respiratory electron-transport chain, because the full inhibition of respiratory electron-transport chains by KCN and HgCl2 abolished the accumulation of gene products. But despite having opposite effects on the redox state of the PQ pools, both inhibitors, KCN and HgCl2, failed to show opposite effects upon the gene expression, indicating redox changes in the PQ pools cannot be the glucose signal for gene accumulation. Furthermore, the cytosolic redox state, as measured by the NAD(P)/H/NAD(P)+ ratio, is not a likely sensor for gene expression because the NAD(P)/H/NAD(P)+ ratio induced by glucose treatment hardly changed under uncoupled conditions. Instead, the glucose effect could be closely related to oxidative phosphorylation or cytosolic pH.

Oxidative Phosphorylation Is Not Involved in the Glucose Induction of Gene Expression—Under coupled conditions, ATP is produced through ATP synthase and there is an increase in pH in the cytosol. Because both cytosolic pH and cytosolic ATP/AMP levels are closely involved in the signaling cascades and translational activation in yeast cells (17), higher plants (22, 34) and the cyanobacterium Synechococcus (23), we hypothesized that either adenylate levels or cytosolic pH may have been regulated in the transient state of the respiratory electron-transport chain, as is the case for some photosynthetic and non-photosynthetic genes (14–16). To test this, we investigated the expression levels of the four genes in various respiratory electron-transfer-defective mutants or WT cells treated with respiratory electron-transfer inhibitors or with a proton uncoupler. Cytobacterial respiratory electron transport shares some electron carriers with photosynthesis, including the PQ and cytochrome (Cyt b6) complex. The cyanobacterial PQ pool is mainly reduced by NADPH dehydrogenase (31, 32) and succinate dehydrogenase (33), and oxidized by the terminal Cyt c oxidase (CtaI) and quinol oxidase (Cyd) (33).

When glucose was fed in the dark to various respiratory electron-transfer-defective mutants, M55 (NADPH dehydrogenase-defective strain), cytochrome c oxidase-defective strain, and quinol oxidase-defective strain, accumulation of transcripts of the four genes was not observed in M55 strains, but was comparable with WT cells in both cytochrome c oxidase-defective and quinol oxidase-defective strains (data not shown).
contribute to regulation of \(\text{crt}\) gene expression. We measured ATP, ADP contents, and ATP/ADP ratio in cells treated with glucose with or without respiratory electron-transport inhibitors or an uncoupler. Glucose treatment for 15 min in the dark reduced both cellular ATP content and ATP/ADP ratio, but electron-transport inhibitors (\(\text{Hgl}_{2}\), KCN), and an uncoupler (CCCP) treatment inhibited this glucose effect (Fig. 4). When \(\text{crt}\) gene transcript abundance was compared with [ATP] and the ATP/ADP ratio, there was no clear linear correlation (data not shown). Therefore, we suggest that the ATP content or ATP/ADP ratio is not closely related to glucose induction of genes expression.

The Increase in Cytosolic pH Is Involved in the Glucose Induction of \(\text{crt}\) Gene Expression—To correlate changes in cytosolic pH with glucose-induced gene expression, we measured the kinetics of acridine yellow fluorescence, an indicator for the cytosolic pH of \textit{Synechocystis} (27). When dark-adapted cells were treated with 10 \(\mu\)M glucose, acridine yellow fluorescence reached a transient peak about 8 min after a 2-min lag period and then remained relatively constant (Fig. 5A). This fluorescence change of acridine yellow was observed only in glucose-treated wild-type cells; it was not induced by other glucose analogs such as L-Glc, OMG, 2-dGlc, and Man, nor in glucose transporter- or glucokinase-defective cells (Fig. 5A).

When wild-type cells were exposed to the chemical inhibitors of either input of electrons to or output from the PQ pool by \(\text{Hgl}_{2}\) and \(\text{CN}^-\) treatment, respectively, or to an uncoupler (CCCP) for 10 min after glucose treatment, acridine yellow fluorescence decreased rapidly to that of dark-adapted cells, abolishing almost fully the glucose effect. However, inactivation of respiratory electron flow to CtaI by DBMIB because of incomplete inhibition of electron flow via Cyt \(b_{d}\) complex resulted in an \(-50\%\) decrease in the steady-state level of acridine yellow fluorescence (Fig. 5B). These data strongly indicate that glucose can modulate cytosolic pH via respiratory electron transport at the expense of NAD(P)H produced from glucose metabolism.

The fluorescence yield of acridine yellow was investigated as a function of CCCP concentration. At 5 \(\mu\)M CCCP, acridine yellow fluorescence decreased to 25\% of the control and then remained relatively constant (Fig. 6A). The dose-dependent inhibitory effect of CCCP also contributed to regulation of \(\text{crt}\) gene expression, with a 50\% reduction in mRNA abundance at concentrations of about 15 \(\mu\)M (Fig. 6, B and C). When the gene expression levels were plotted against the acridine yellow fluorescence yields, expression of the four genes increased with increasing acridine yellow fluorescence, showing saturation above 25\% of acridine yellow fluorescence (Fig. 6D).

**DISCUSSION**

Light induction of carotenoid biosynthesis is a widespread phenomenon, but the light regulation mechanism of carotenoid gene expression is not clear. Photosynthetic redox control (35) or control by red- and blue-light photoreceptor systems (4, 6) over carotenoid biosynthesis genes have been reported in green algae and higher plants. In \textit{Synechocystis}, expression of both \(\text{crtB}\) and \(\text{crtP}\) genes is light-dependent, but little is known about light signaling (10). In the present study, in addition to \(\text{crtB}\) and \(\text{crtP}\) genes (10), we observed light induction of another carotenogenesis gene, \(\text{crtQ}\), as well as \(\text{crtR}\), in a dark-to-light shift (Fig. 1). Thus, all four carotenoid biosynthesis genes are light-regulated; this is atypical, as often only a few genes, such as \(\text{crtB}\), are tightly regulated at the level of transcript abundance, while \(\text{crtQ}\) mRNA often shows little variation (3).

However, light is not the sole signal for the accumulation of \(\text{crt}\) gene mRNA because glucose alone in the dark results in a similar increase (Fig. 1). The glucose induction is apparently related to the transcriptional activity rather than to the stability of mRNAs as indicated by rifampicin treatment. Glucose induction in yeast, higher plants, and human pancreatic cells of a range of genes is mediated by a membrane-bound transporter homolog, a hexokinase, or extensible metabolism of phosphorylated glucose (18, 36). For \textit{Synechocystis}, metabolism of phos-
Cytosolic pH and crt Expression in Synechocystis

In this respect, cyanobacterial glucose-sensing is analogous to insulin secretion in pancreatic a cells, where ATP generation with a concomitant decrease in ADP concentration by glycolytic and oxidative events from NADH represent a key transduction phenomenon (19, 20). If this is the case in cyanobacteria, the inhibition of respiratory electron flow or oxidative phosphorylation would diminish the glucose effect, because NAD(P)H produced by means of the oxidative pentose phosphate pathway and glycolysis donates electrons to the component of respiratory electron transport in cyanobacteria (PQ) (13) to drive ATP formation. Indeed, chemical inhibition of either inputs (Hg2+ treatment) or outputs (CN− treatment) of electrons to the PQ pool or uncoupling of proton gradient (for CCCP treatment, see Ref. 37) fully diminished the glucose effect (Fig. 3). Partial blockage of the electron flow (to Cyt c oxidase) by DBMIB (27) inhibited the early induction (at 15 min) of the four genes by glucose (Fig. 3), which could have resulted from the partial blockage of proton pumping. In line with this view, genetic inhibition of electron input into the PQ pool (in NADPH dehydrogenase-defective cells) or output to CtuI- and Cyl-defective cells fully or partially inhibited the glucose induction of the four genes (data not shown). Further, the fact that uncoupling conditions, where oxidation of NAD(P)H still occurred without ATP generation, inhibited glucose induction (Fig. 3), strongly suggesting that both the redox state of the cytosol (indicated by the NAD(P)/RNA(D/P ratio) and of the PQ pool of photosynthetic membranes is not likely to be an important factor for glucose induction of crt genes. Moreover, the observation that changes in ATP content and the ATP/ADP ratio are not closely related to the extent of gene expression (data not shown) indicates that energy charge or adenylate status is not likely to be involved in the transcriptional activation of the four genes; this is different from the case of yeast cells, where selective gene expression induced by glucose is sensed via adenylate contents (17).

If cytosolic, membrane redox state, ATP content, or ATP/ADP ratio are not determining factors for gene expression, then alkalinization of the cytosol could be a decisive factor, as respiratory electron transport can provide not only ATP but also an alkaline cytosolic environment for a variety of cellular processes. Indeed, glucose treatment in the dark induced alkalinization of cytosol via respiratory electron transport; the alkalinization was glucose-specific (Fig. 5A) and required further metabolism of glucose phosphorylation via respiratory electron transport (Fig. 5B). The extent of expression of the four genes showed a saturation curve with an increasing acidic yellow fluorescence yield (Fig. 6D). Although it is complicated to draw a simple relationship between fluorescence yield and intracellular pH because of unspecified indicator binding to DNA, proteins, and lipids (27), one may expect that a slight change of cytosolic pH is enough to saturate gene expression. It was suggested that illumination of cyanobacterial cells with saturating light increased cytosolic pH by about 0.5 units (27). Because the extent of the fluorescence increase by glucose treatment (∆F/Fo = 0.85) was about half of that by saturating light intensity (∆F/Fo = 1.75) in this experiment, glucose treatment could increase the cytosolic pH by about 0.25 units. Above 25% of acidic yellow fluorescence, the extent of gene expression was saturated (Fig. 6D). To confirm that the changes in the intracellular pH would affect the expression of crt genes, we had tried other uncouplers such as nigericin and NH4Cl (37). In the presence of 10 mM KCl, nigericin indeed inhibited the alkalinization of cytosol. But the concentration required to fully abolish the cytosolic pH increase to the same extent as that obtained by CCCP was over 1 mM (data not shown). As this concentration is expected to be high enough to evoke some
harmful side effects, we did not follow up further. For the NH₄Cl, we did not use it either, as NH₄Cl treatment of the intact wild-type cells in the dark increased the cytosolic pH (contrary to our expectations; data not shown), probably because of its being utilized as a nitrogen source instead of acting as an uncoupler.

Based on our results, we propose a working model for the glucose induction of carotenoid biosynthesis genes in *Synechocystis* as shown in Fig. 7. When dark-adapted cells are exposed to exogenously supplied glucose, up-regulation of *crt* genes are induced. This glucose induction is mediated by respiratory electron flow coupled to cytosolic alkalization, instead of respiratory electron redox, cytosolic redox status, or ATP/ADP ratio. In animals, it has been known for quite some time that cytosolic pH changes are associated with changes in cellular metabolism and development, such as fertilization, spore germination, cell cycle, and insulin stimulation (21). In plants, cytosolic pH is known to act on a wide range of cellular enzymes, membrane transport, and regulatory agents (21). As a regulatory agent, ΔpH has been considered to be a synergistic messenger that acts as a master metabolic variable through which the actions of other effectors, such as calcium signaling in differentiation and cellular proliferation (21), are integrated. In this respect, cytosolic pH is established as a second messenger of ABA signaling in guard cells of higher plants (34). For the gene expression of chloroplasts, the proton gradient formed during electron transport is known to regulate translation elongation in chloroplasts (22). Thus, it is expected that the cytosolic pH of *Synechocystis* will activate gene transcription through either activation of transcriptional control proteins or the deactivation of transcriptional repressors. However, the precise mechanism by which cytosolic alkalization regulates the expression of carotenoid genes remains unclear.

In conclusion, we have demonstrated that expression of the four carotenoid biosynthesis genes, *crtB*, *crtP*, *crtQ*, and *crtR*, in *Synechocystis* is dependent upon exogenously supplied glucose. Glucose metabolism is involved in the glucose induction of these four genes, which is presumably sensed by pH changes in cytosol. Direct evidence for the dependence of the expression of carotenoid biosynthesis genes upon the cytosolic pH and the gene activation mechanisms remains to be determined.

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