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***Review***

# Physiological and Molecular Aspects of the Inorganic Carbon-Concentrating Mechanism in Cyanobacteria[1]

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## ABSTRACT

**This paper reviews progress made in elucidating the inorganic carbon concentrating mechanism in cyanobacteria at the physiological and molecular levels. Emphasis is placed on the mechanism of inorganic carbon transport, physiological and genetical analysis of high-$CO_2$-requiring mutants, the polypeptides induced during adaptation to low $CO_2$, the functional significance of carboxysomes, and the role of carbonic anhydrase. We also make occasional reference to the green algal inorganic carbon-concentrating mechanism.**

Many photosynthetic microorganisms possess a mechanism for active intracellular accumulation of $C_i$[2] that enables them to compensate for the 5- to 20-fold difference (in green algae and cyanobacteria, respectively) between the $CO_2$ concentration in their environment and the $K_m(CO_2)$ of their Rubisco. The activity of the $C_i$-concentrating mechanism increases during adaptation from high to low external $CO_2$ concentrations, one of a syndrome of changes that lead to an elevated apparent photosynthetic affinity for extracellular $C_i$. This review focuses on certain aspects of the $C_i$-concentrating system currently under active investigation. Although rigid limitation of space obliges us to confine ourselves largely to cyanobacteria, we also occasionally refer to green algae in cases in which progress has recently been made relevant to the topic discussed (for earlier reviews, see refs. 1, 2, 7, 13–15, 20).

## MECHANISM OF $C_i$ UPTAKE

The intracellular level of $C_i$, at the steady-state of photosynthesis, is considerably higher than could be accounted for by the passive equilibration of $CO_2$ and $HCO_3^-$ across the cell membrane, indicating active transport (2, 13, 15). In *Anabaena,* supply of $C_i$ triggers immediate (13) but transient hyperpolarization, and evidence has been presented that a primary electrogenic pump is involved in the $C_i$ transport mechanism. It is not yet known whether a primary bicarbonate pump is operating; the pump might establish a transmembrane electrochemical gradient for some other ion as the immediate source of energy for $C_i$ uptake. The demonstrated $Na^+$ requirement for $HCO_3^-$ uptake (see below) suggests that uptake might be a secondary active $Na^+$ symport, driven by a transmembrane $Na^+$ gradient established by a $Na^+$ extrusion pump. Alternatively, particularly at pH values below 7.0, proton symport driven by the protonmotive force generated by the $H^+$ pump could be envisaged, although this would demand a stoichiometry greater than 1:1 (12, 13).

A role for $Na^+$ in the $C_i$ uptake mechanism was suggested by its highly specific effect on apparent photosynthetic affinity for external $C_i$ and on the $K_m(HCO_3^-)$ of the $C_i$ transport system (12, 13, 15). The effect of $Na^+$ is far larger in the case of $HCO_3^-$ than is $CO_2$ uptake, but only micromolar $Na^+$ concentrations are required to achieve the maximal effect on $CO_2$ uptake; millimolar $Na^+$ concentrations are required in the case of $HCO_3^-$ (12, 15). For some as yet unknown reason, $HCO_3^-$ uptake in nonaerated cultures of *Synechococcus* is not $Na^+$ dependent (15). Three alternative models to account for the $Na^+$ effect on $HCO_3^-$ uptake have been considered, but it has not yet proved possible to distinguish among them experimentally (12). The situation is complicated by the fact that the $C_i$-dependent transient hyperpolarization (Kaplan in 13, 14) would affect the driving force for ions other than those being pumped, as well as the gating of voltage-dependent ion channels. This complexity underlines the desirability of carrying out experiments with plasmalemma-enriched membrane vesicles where the solutions at both membrane interfaces are controlled. Functioning vesicles have not yet been isolated from cyanobacteria, nor have attempts to demonstrate $C_i$- or $Na^+$-dependent ATPase activity in isolated membrane fractions been successful.

In cyanobacteria, mediated transport is indicated for both $CO_2$ and $HCO_3^-$ (3, 15). The kinetic parameters for photosynthetic rate *versus* intracellular $C_i$ concentration indicated that no matter which $C_i$ species is supplied, $HCO_3^-$ appears to be the form that arrives at the cytoplasmic surface of the plasmalemma (Kaplan in 14). This observation suggests that the membrane transport proteins involved may serve as vectorial carbonic anhydrases (2, 13, 22). Because $CO_2$ is the substrate for Rubisco, this deduction points to the likelihood of a role for CA in the flow of $C_i$ to the carboxylation reaction. The uncatalyzed formation of $CO_2$ from the internal $C_i$ pool has,

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[2] Abbreviations: $C_i$, inorganic carbon; CA, carbonic anhydrase; *rbc,* the cyanobacterial operon encoding the large and small subunits of Rubisco.

in fact, been estimated as too slow to account for the rate of $CO_2$ fixation (15, 22, 24). Use of the mass spectrometer has significantly improved the detection of the very low CA activity observed in cyanobacteria (in comparison with green algae) and, thus, the occurrence of CA in cyanobacteria is now firmly established (2, 15).

There is still controversy as to whether a common system or separate systems transport the two $C_i$ species (2, 12, 15). On the basis of ethoxyzolamide inhibition of $C_i$ transport, it has been proposed (2) that dehydration of $HCO_3^-$ and rehydration of $CO_2$ are successive steps during transport, suggesting a common carrier for $CO_2$ and $HCO_3^-$. On the other hand, the differential $Na^+$ effect on $CO_2$ and $HCO_3^-$ transport (15) suggests the presence of separate systems for the $C_i$ species. Attempts to resolve this conflict by using carbon oxysulfide to inhibit $C_i$ uptake have led to contradictory conclusions (3, 15). The relative roles of $CO_2$ and $HCO_3^-$ during steady-state $C_i$ uptake may vary considerably between species, accounting for some of the controversy regarding the dominant $C_i$ species taken up.

The rate of $C_i$ uptake is very strongly depressed in the dark or in the presence of inhibitors of photosynthetic electron transport (2, 13). PSI energy is very probably involved in driving $C_i$ transport (2). Light is required not only for energization but also for a time-dependent activation of the transport system (13). In contrast to energization, activation requires PSII activity, but only at a very low level. Recent pioneering studies by Miller *et al.* (in 7) revealed strong correlations between the rates of $CO_2$ uptake and of PSII fluorescence quenching, and also between the extent of the latter and the size of the internal $C_i$ pool. The mechanism(s) involved are not yet understood.

## HIGH-$CO_2$-REQUIRING MUTANTS AS A TOOL FOR ELUCIDATING THE $C_i$ CONCENTRATING MECHANISM

Mutants defective in $C_i$ transport and accumulation ability could clearly serve as tools for the elucidation of the $C_i$ transport mechanism. An approach that has proved useful for detecting mutants defective in such ability is selection for high-$CO_2$ requirement for growth. Mutants were obtained by chemical mutagenesis as well as by site-specific mutations. Most of the cyanobacterial high-$CO_2$-requiring mutants isolated, however, were found to be capable of efficient $C_i$ transport and to be deficient in some other respect (see Lieman-Hurwitz *et al.* in 7). Two transport mutants, RKa and RKb, have been successfully isolated by Ogawa (18) in the case of *Synechocystis* PCC6803. In RKa, the polypeptide deduced from the sequence of the relevant gene shows very high homology to subunit II of NADH dehydrogenase from tobacco, and it was suggested that this enzyme is a component of the $C_i$ transport system. It remains to be seen whether the NADH dehydrogenase is involved in the energization or the activation of $C_i$ transport and whether this enzyme is also involved in the transport of other metabolites in cyanobacteria. The polypeptide (80 amino acids) deduced from the sequence of the open reading frame that complemented the mutation in RKb did not reveal any significant homology to a known protein. A hydropathy plot indicated that it contains two hydrophobic regions (21 and 22 amino acids long), suggesting a transmembrane protein, but its cellular location in the cyanobacterial plasma membrane (the expected site of the putative $C_i$ transport mechanism) still needs to be demonstrated.

A 42-kD polypeptide accumulates in the cytoplasmic membrane fraction isolated from low-$CO_2$ grown, but not high-$CO_2$ grown, cells of *Synechococcus* sp. PCC7942 (19), suggesting a possible role in $C_i$ transport. However, inactivation of the gene encoding this polypeptide does not affect ability to grow under low-$CO_2$ and to concentrate $C_i$ internally (19). The high-$CO_2$-requiring mutant 0221 provides useful confirmatory evidence that the 42-kD polypeptide is not directly involved in $C_i$ transport. This mutant does not accumulate the 42-kD polypeptide but, nevertheless, manifests almost unimpaired $C_i$ uptake capability (8). It is of some interest that ability to accumulate the polypeptide has been affected, because the mutation in 0221 has been identified within an open reading frame in the 5′-flanking region of *rbc* (8), and Southern analysis indicates that *cmpA*, the gene encoding-42-kD polypeptide, is located in a different genomic region (8, 19). This may indicate the presence of regulatory sequences in the region containing the lesion in this mutant. Sequence analysis indicates similarity between the 42-kD polypeptide and a carotenoid-binding protein that is synthesized during high light treatment (23). Thus, its accumulation during adaptation both to low $CO_2$ and to high light might be a consequence of the photoinhibitory conditions experienced by the cells following the exposure to low $C_i$.

## THE FUNCTIONAL SIGNIFICANCE OF THE CARBOXYSOMES (AND PYRENOIDS?)

A common property shared by many of the high-$CO_2$-requiring mutants characterized so far is the aberrant nature, or the complete absence, of their carboxysomes. Carboxysomes are polyhedral bodies that have been widely observed in cyanobacteria as well as in certain autotrophic bacteria (5). They contain most of the Rubisco in the cell and are surrounded by a protein shell. Because phosphoribulose kinase and triose phosphate dehydrogenase are located outside the carboxysomes, photosynthesis in cyanobacteria must involve the fluxes of RuBP and 3-PGA into and out of the carboxysomes. The high-$CO_2$-requiring mutants displaying aberrant or absent carboxysomes exhibit a very low apparent photosynthetic affinity for external $C_i$, two orders of magnitude lower than that of the wild type. This apparently low affinity does not stem from inability to accumulate $C_i$ internally, because the size of the internal $C_i$ pool does not differ from that in wild-type cells. Neither can it be accounted for by alterations in the kinetic parameters for activated Rubisco with respect to $CO_2$ (8). This observation suggests inability to utilize the internal $C_i$ pool efficiently, possibly due to a low $CO_2$ concentration at the carboxylation site within the carboxysomes.

Further evidence indicating that the carboxysomes may play a role in the $C_i$-concentrating mechanism and suggesting, in addition, that the native cyanobacterial Rubisco is involved in their functional organization was provided by Pierce *et al.* (21). They replaced the *rbc* in *Synechocystis* PCC6803 with the corresponding gene from *Rhodospirillum rubrum*. The

mutant ("cyanorubrum") lacks visible carboxysomes and requires high $CO_2$ for growth, although it is capable of accumulating $C_i$. It is not improbable that the absence of carboxysomes in cyanorubrum may result from the differing structure of *Rhodospirillum* Rubisco, which lacks small subunits. The role of the small subunit in the functioning of Rubisco is not known, but it is thought to play an important part in the organization of the holoenzyme and may have a major role in the structural organization of the carboxysome. In *Thiobacillus,* the small subunits appear to be associated with the carboxysomal shell. In the high-$CO_2$-requiring mutant of *Synechococcus* PCC7942, EK6, the native *rbcS* has been replaced by a modified one (84 nucleotides longer), thus encoding for a larger small subunit (by approximately 3-kD [Lieman-Hurwitz *et al.* in 7]). The carboxysomes in the mutant are less susceptible to breakage in the French press, as indicated by the retention of the Rubisco activity in the carboxysomal fraction, in contrast to the case of the wild type, in which approximately half the activity appears in the supernatant. The apparent photosynthetic affinity for external $C_i$ has been decreased 50-fold in the mutant. This low affinity is attributable to the observation that, in the mutant, the Rubisco becomes fully activated only when the cells are exposed to higher $CO_2$ concentrations than in the case for the wild type.

A quantitative model for $C_i$ fluxes and photosynthesis in cyanobacteria has been proposed (24) assigning a crucial role to the carboxysomes. It has been recognized that back-diffusion of $CO_2$ from the accumulated $C_i$ pool within the cell could result in a prohibitively high energy cost for a $C_i$ concentrating mechanism (see Raven and Lucas in 14), and it has therefore been assumed that the plasmalemma must have a very low permeability coefficient for $CO_2$. Such low permeability, however, runs counter to our knowledge of the properties of polar lipid bilayers. The model (24) transfers the putative $CO_2$ diffusion barrier in the cell from the plasmalemma to the surface of the carboxysomes. It postulates that CA is absent from the cytoplasm and that $HCO_3^-$ and $CO_2$ do not reach equilibrium in this compartment. The accumulated $HCO_3^-$ ions penetrate into the carboxysomes, where the presence of CA at low concentration leads to $CO_2$ generation and subsequent fixation by Rubisco. $CO_2$ fixation rates predicted by this model accord well with experimentally observed rates (22, 24).

Further development of the model applying equations based on three-dimensional diffusion (Reinhold *et al.* in 7) suggests that it may be possible to dispense with the requirement for a substantial barrier to $CO_2$ in the cell other than that constituted by the closely packed Rubisco molecules within the carboxysomes. If the CA is placed well in the interior of the carboxysome, much of the $CO_2$ generated will be fixed as it diffuses outward past Rubisco sites along the (possibly tortuous) diffusion path. Thus, the model suggests that part of the biological significance of the packing of Rubisco into carboxysomes is the barrier to $CO_2$ diffusion that such an arrangement affords. The model further demonstrates that diffusion resistances of this order would not give rise to steep diffusion gradients in the opposite direction for the essential substrates ($HCO_3^-$ and RuBP) that must diffuse into the carboxysome from the cytoplasm, owing to the fact that solutes diffusing from the periphery of a sphere to its center are strongly concentrated as they go.

The organization of the carboxysome may thus ensure a highly efficient means for generation and utilization of $CO_2$ at closely contiguous sites; the dense packing of the Rubisco provides a barrier to escaping $CO_2$. It is tempting to speculate that pyrenoids fulfill the same highly organized function in green algae.

Price and Badger (22) have devised an elegant test of one aspect of the model. They expressed human CA in *Synechococcus* cells and noted that the cells became high-$CO_2$-requiring because they had lost the ability to accumulate internal $C_i$. They concluded that the latter ability depended on the absence of CA from the cytosol and its specific location in the carboxysomes. A number of other reports have localized CA in the pelletable fraction (22, 24), which would be consistent with its association with the carboxysomes and absence from the cytoplasm as proposed in the model. Clearly, cloning of the cyanobacterial CA gene and isolation of mutants defective in CA activity will provide the necessary means to determine the cellular location and role of CA in cyanobacterial photosynthesis, but this has not been accomplished yet. A mutant defective in CA might be expected to demand high-$CO_2$ for growth, and the desired mutant might therefore be present among the high-$CO_2$-requiring mutants isolated in various laboratories. Bedu *et al.* (4) have obtained a mutant resistant to CA inhibitors that might be used to identify the relevant gene. They also reported that CA activity in *Synechocystis* is very low under high external $C_i$ and is stimulated by $C_i$ limitation during growth.

Significant progress has recently been achieved in elucidating the role of CA in the $C_i$-concentrating mechanism in green algae. Both a periplasmic and an intracellular CA are present in *Chlamydomonas*; the level and activity of the former increase during adaptation to low $CO_2$, whereas the latter is constitutive (16). Analysis of the 37-kD soluble polypeptide identified as periplasmic CA (6, 9) led to the suggestion that the holoenzyme is composed of two large (35- and 36.5-kD) and two small subunits (4-kD, Fukuzawa *et al.* in 7). Expression of the relevant genomic region depends both on the presence of low ambient $CO_2$ concentration and on photosynthetic electron transport. These findings provide the molecular basis for the observed $CO_2$ and light dependence of the periplasmic CA activity (9). *Chlamydomonas* mutants containing lesions in the ca-1 locus, and thought to be defective in intracellular CA activity, accumulate $C_i$ to higher levels than does the wild type, but demand high $CO_2$ for growth (9, Spalding *et al.* in 7). The relatively low intracellular CA level and possible contamination by the relatively abundant periplasmic CA has made difficult a firm conclusion as to whether the former is located in the cytoplasm or chloroplast. This question has considerable importance for modeling the $C_i$ concentrating mechanism in green algae. Various models have been proposed (2) that are beyond the scope of the present review; they may have to be reconsidered in view of the recent demonstration that Rubisco and CA are present in the pyrenoid (see McKay and Gibbs, Kuchitsu *et al.* in 7). By analogy with the cyanobacterial cell as visualized above, the internal CA might be confined to the pyrenoid, as is Rubisco. The functional significance of this organization might

be central for our understanding of the $C_i$-concentrating mechanism.

## THE NATURE OF THE SIGNAL FOR ADAPTATION TO LOW $CO_2$ CONCENTRATION AND ITS MOLECULAR BASIS

The nature of the signal that induces the syndrome of changes characteristic of adaptation to low $CO_2$ is one of the interesting problems still not resolved. Adaptation to low $CO_2$ in both the cyanobacterium *Anabaena* and the green alga *Chlamydomonas* is faster the lower the ratio $CO_2/O_2$ concentration, and is a function of this ratio rather than of $CO_2$ concentration as such (26). Therefore, it was suggested that a metabolite in the glycolate pathway is involved in signal perception. This possibility might be investigated with the aid of high-$CO_2$-requiring mutants defective in phosphoglycolate phosphatase activity such as the *Chlamydomonas* mutant 18–7F (26).

Badger (2) reported that in *Synechococcus* the extent of adaptation is a function of the total $C_i$ concentration in the medium. It is conceivable that a periplasmic protein senses ambient $C_i$ level, but such a protein has not been identified, in contrast to the case of sulfate uptake, in which a periplasmic protein has been implicated in the perception of the presence of sulfate in the medium (11). Several $CO_2$-dependent promoters have recently been detected in *Synechococcus* (25). The nature of these promoters, the elements involved in their induction, and the genes that they regulate are not known. It is important to note that, in the cases of *rbc* and *cmpA*, the expression of both of which depends on the level of $CO_2$, the promoter regions contain three highly homologous boxes. It remains to be seen whether these boxes serve as regulatory sequences, involved in the response to the $CO_2$ concentration itself, or to a metabolite the level of which is affected by the concentration of $C_i$.

Induction of the low-$CO_2$ syndrome has been correlated with the synthesis of a number of polypeptides, both soluble and membrane bound. In cyanobacteria, the level of the 42-kD polypeptide increases during adaptation to low-$CO_2$ (discussed above). No other polypeptide has yet been shown to respond to low $CO_2$ level, although the number of carboxysomes increases (27). In *Chlamydomonas*, on the other hand, the levels of several polypeptides have been shown to rise during adaptation to low $CO_2$ (10, 17, 26), including the 37-kD (the periplasmic CA, see above). Specific functions have not yet been identified for the other polypeptides, including the 44- and 46-kD polypeptides missing in the high-$CO_2$-requiring transport mutant pmp-1. That the latter can respond to the $CO_2$ level is indicated by its ability to accumulate the adaptation-related 20- and 37-kD polypeptides (17). Control by ambient $CO_2$ level of the synthesis of a 36-kD polypeptide appears to be exerted via regulation of mRNA abundance. This membrane-associated polypeptide (apparently chloroplast-, but not thylakoid-located) is distinct from the periplasmic CA as shown by use of antibodies (10).

Mutants defective in adaptation ability might be useful for clarification of the molecular basis of adaptation to the ambient $CO_2$ level. Such mutants would be expected to exhibit normal photosynthetic performance under high-$CO_2$ conditions (including apparent photosynthetic affinity for $C_i$), but would fail to adapt and grow under low $CO_2$. This behavior contrasts with that of other types of high-$CO_2$-requiring mutants defective in the ability to utilize the internal $C_i$ pool, which exhibit very low apparent photosynthetic affinity to $C_i$ (8). The *Chlamydomonas* mutants CIA-5 (which does not synthesize any of the adaptation-related polypeptides discussed above, 17) and 18–7F (defective in phosphoglycolate phosphatase, 26) fulfill these requirements. So does the *Synechococcus* mutant D4, which was constructed by replacing the 1.4 kilobase *PstI* fragment downstream of *rbc* with a kanamycin resistance cartridge (Lieman-Hurwitz *et al.* in 7). In this mutant, the lesion affects purine biosynthesis, another $CO_2$-dependent pathway. Therefore, this photosynthetic characteristic does not exclusively indicate a lesion in a gene directly involved in adaptation to low $CO_2$.

The lesions in many of the high-$CO_2$-requiring mutants of *Synechococcus* so far investigated have been mapped in *rbc* itself or in its flanking regions (see Lieman-Hurwitz *et al.* in 7). The level of some of the transcripts originating from this region (including that from *rbc*) depends on the $CO_2$ concentration during growth. This strongly suggests that the genomic region of *rbc* contains a cluster of genes involved in the ability to grow at low ambient $CO_2$, and the question of the regulation of these genes will have to be addressed.

## PROSPECTS AND PERSPECTIVES

The employment of recombinant DNA techniques combined with physiological and genetical characterization of mutants has already brought about significant progress. Further use of this approach is likely to lead to the identification of the genes encoding the polypeptides appearing during adaptation of green algae and clarification of their role. It is also likely to lead to the identification of the genes in the cluster proposed for *Synechococcus* and the mode of their regulation. It is important to find out whether similar clusters are also indicated in other organisms and whether other genes involved in the $C_i$-concentrating mechanism are also clustered. Clarification of the possible functional association between CA and Rubisco in the carboxysome is likely to follow the cloning and modification of the cyanobacterial CA gene. In view of the functional advantage of having Rubisco and CA organized in close contiguity, as emphasized in the quantitative carboxysome model, a search is likely to be made for functional analogy between pyrenoids and carboxysomes. With the isolation of more mutants defective in $C_i$ transport, it will be possible to identify the genes and proteins involved, to clarify the nature of the primary pump, and to determine whether separate or common carrier systems transport bicarbonate and $CO_2$ and, in green algae, their location. Isolation of functional plasma membrane vesicles should help to clarify the role of $Na^+$ as well as other physiological questions related to the mechanism of $C_i$ transport, its energization, and its activation.

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