Bicarbonate Binding Activity of the CmpA Protein of the Cyanobacterium *Synechococcus* sp. strain PCC 7942 Involved in Active Transport of Bicarbonate*

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Shin-ichi Maeda‡§, G. Dean Price‡§, Murray R. Badger‡§, Chika Enomoto¶, and Tatsu Ozumata||

From the ‡Molecular Plant Physiology Group, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra ACT 2601, Australia and the §Laboratory of Molecular Plant Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

The *cmpABCD* operon of the cyanobacterium *Synechococcus* sp. strain PCC 7942 encodes an ATP-binding cassette transporter involved in HCO$_3^-$ uptake. The three genes, *cmpB*, *cmpC*, and *cmpD*, encode membrane components of an ATP-binding cassette transporter, whereas *cmpA* encodes a 42-kDa cytoplasmic membrane protein, which is 46.5% identical to the membrane-anchored substrate-binding protein of the nitrate/nitrite transporter. Equilibrium dialysis analysis using H$^{14}$CO$_3^-$ showed that a truncated CmpA protein lacking the N-terminal 31 amino acids, expressed in *Escherichia coli* cells as a histidine-tagged soluble protein, specifically binds inorganic carbon (CO$_2$ or HCO$_3^-$). The addition of the recombinant CmpA protein to a buffer caused a decrease in the concentration of dissolved CO$_2$ because of the binding of inorganic carbon to the protein. The decrease in CO$_2$ concentration was accelerated by the addition of carbonic anhydrase, indicating that HCO$_3^-$, but not CO$_2$, binds to the protein. Mass spectrometric measurements of the amounts of unbound and bound HCO$_3^-$ in CmpA solutions containing low concentrations of inorganic carbon revealed that CmpA binds HCO$_3^-$ with high affinity ($K_d$ = 5 μM). A similar dissociation constant was obtained by analysis of the competitive inhibition of the CmpA protein on the carboxylation of phosphoenolpyruvate by phosphoenolpyruvate carboxylase at limiting concentrations of HCO$_3^-$ transfer. These findings showed that the *cmpA* gene encodes the substrate-binding protein of the HCO$_3^-$ transporter.

Cyanobacteria possess multiple transporters for uptake of inorganic carbon (CO$_2$ and HCO$_3^-$; designated C$_i$) into the cell. The cells accumulate C$_i$ in the cytoplasm as HCO$_3^-$ and convert it into CO$_2$ in carboxysomes, the polyhedral inclusion bodies to which ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is localized, to elevate the CO$_2$ concentration around the CO$_2$-fixing enzyme (1, 2). The transport of inorganic carbon has been the least understood step of carbon assimilation in cyanobacteria. We have, however, recently identified a high affinity HCO$_3^-$ transporter (BCT1) of *Synechococcus* sp. strain PCC 7942 (3). The transporter is encoded by the four genes *cmpA*, *cmpB*, *cmpC*, and *cmpD* (4, 5), which form a low CO$_2$-inducible operon (3). The *cmpA*, *cmpB*, *cmpC*, and *cmpD* genes are strongly similar to the genes encoding the nitrate/nitrite transporter, *nrtA*, *nrtB*, *nrtC*, and *nrtD*, respectively, of the same organism (6–8). Similar to *nrtB*, *cmpB* encodes a hydrophobic protein with structural similarities to the integral membrane components of ABC transporters; *cmpC* and *cmpD* encode the ATP-binding cassette proteins strongly similar to *nrtC* and *nrtD*, respectively. The product of *cmpA* is a 42-kDa cytoplasmic membrane protein, which is 46.5% identical to the *nrtA* gene product that functions as the membrane-anchored substrate (nitrate and nitrite)-binding protein (9). The similarity of *cmpA* to NrtA and its involvement in HCO$_3^-$ uptake strongly suggest that the product of *cmpA* is the substrate-binding protein of the HCO$_3^-$ transporter. In this work, we have biochemically verified this assumption by showing high affinity binding of HCO$_3^-$ to a recombinant CmpA protein. Although contamination by atmospheric CO$_2$ made it impossible to determine the dissociation constant of CmpA and HCO$_3^-$ by the equilibrium dialysis technique using H$^{14}$CO$_3^-$, use of a mass spectrometer enabled measurements of the amounts of free and bound H$^{14}$CO$_3^-$ in CmpA solutions containing low concentrations of H$^{14}$CO$_3^-$, allowing kinetic analysis of binding of HCO$_3^-$ to the protein. The dissociation constant was estimated also from the competitive inhibition of CmpA on the phosphoenolpyruvate carboxylase (PEPCase) reaction in the presence of limiting amounts of HCO$_3^-$; Both methods resulted in dissociation constant of 5 μM for CmpA and HCO$_3^-$, demonstrating that the *cmpA* gene encodes a HCO$_3^-$-binding protein.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant CmpA—A 1.3-kilobase pair DNA fragment, carrying a truncated *cmpA* coding region lacking the first 93 bases, was cloned between the BamHI and Smal sites in the polylinker of the expression vector pQE-32 (Qiagen). The resulting plasmid (pQECMPA) carried a chimeric gene, which encodes a fusion protein consisting of an N-terminal amino acid segment carrying six consecutive histidine residues (MRGSH6GI) and truncated CmpA lacking the histidine-tagged protein was purified on Ni$_2^+$- (or Co$_2^+$-) iminodiacetic acid resin (10). The purified protein solution was dialyzed for 18 h at 15 °C against a buffer containing 20 mM TES-NaOH, pH 7.5, and 100 mM NaCl, with continuous sparging with N$_2$ gas.
Assay of C\textsubscript{4} Binding Using H\textsuperscript{14}CO\textsubscript{3}—Analysis by equilibrium dialysis, using H\textsuperscript{13}CO\textsubscript{3}, of the binding of C\textsubscript{4} to the recombinant CmpA protein was performed at 30 °C in a buffer containing 20 mM sodium phosphate, pH 8.0, and 100 mM NaCl. 1.36 ml aliquots of protein solution (8 mg/ml) were dialyzed against various volumes of a buffer containing 0.375 M TES-NaOH, pH 7.5, and 100 mM NaCl at 30 °C for 72 h. Lower the C\textsubscript{4} concentration in buffer, the reaction was initiated by the sparging of N\textsubscript{2} gas into the cuvette. The rate of reaction was determined in the presence and absence of NADH, NADPH, and protein samples were subjected to electrophoresis in the buffer system of Laemmli (14), polypeptides were stained with Coomassie Brilliant Blue. Protein concentration was determined with a bicinchoninic acid assay (Pierce). Specific Binding of C\textsubscript{4} to the Recombinant CmpA Protein—Table I shows the distribution of 14C between 1.36 ml aliquots of the solution of the recombinant CmpA (8 mg/ml) and the dialysis buffer after equilibrium dialysis with NaH\textsuperscript{14}CO\textsubscript{3}. Under the experimental conditions, the protein solution contained 64% higher levels of 14C than the dialysis buffer. The accumulation of 14C in the protein solution was abolished by an excess amount of NaH\textsuperscript{13}CO\textsubscript{3} but not with equivalent concentrations of NaHCO\textsubscript{3}, Na\textsubscript{2}CO\textsubscript{3}, and Na\textsubscript{2}SO\textsubscript{3}, indicating that the truncated CmpA specifically binds C\textsubscript{4}. In parallel experiments with no added CmpA and NaH\textsuperscript{14}CO\textsubscript{3}, however, mass spectrometric analysis showed that the solutions in the dialysis cells contain 150–200 μM of 13C, due to contamination by atmospheric CO\textsubscript{2}. Kinetic analysis of the binding of C\textsubscript{4} to the recombinant CmpA at low C\textsubscript{4} concentrations (<100 μM) was thus problematical by this method.

The C\textsubscript{4} Species That Binds to the CmpA Protein—A mass spectrometer assay was employed to determine the C\textsubscript{4} species that binds to CmpA protein. When 0.5 ml of buffer containing 0.040 μM of CO\textsubscript{2} (1 μM total C\textsubscript{4} CO\textsubscript{2}) was added to 2.5 ml of the same buffer containing 6 μM CO\textsubscript{2} (170 μM total C\textsubscript{4}) the concentration of the dissolved CO\textsubscript{2} decreased by 1.0 μM in 25 s (Fig. 2A). CA did not affect the rate of decrease in the CO\textsubscript{2} concentration (Fig. 2A), indicating that injection of buffer did not cause disequilibrium of the CO\textsubscript{2} to HCO\textsubscript{3} ratio and that the CO\textsubscript{2} decrease was due solely to C\textsubscript{4} dilution. After injection of 0.5
TABLE I

<table>
<thead>
<tr>
<th>Competitive substrate</th>
<th>14C bound in the protein solution (A)</th>
<th>14C bound in the dialysis buffer (B)</th>
<th>A - B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.70</td>
<td>4.08</td>
<td>2.62</td>
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<td>NaH14CO3</td>
<td>4.88</td>
<td>4.04</td>
<td>0.84</td>
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<tr>
<td>NaN3</td>
<td>6.55</td>
<td>4.03</td>
<td>2.52</td>
</tr>
<tr>
<td>NaN2O</td>
<td>6.41</td>
<td>3.91</td>
<td>2.50</td>
</tr>
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<td>6.67</td>
<td>4.15</td>
<td>2.52</td>
</tr>
<tr>
<td>Na2HCO3</td>
<td>6.15</td>
<td>3.72</td>
<td>2.43</td>
</tr>
</tbody>
</table>

Fig. 2. Disequilibrium of CO2 and HCO3 caused by binding of C1 to CmpA. A, effects of carbonic anhydrase on the changes in the CO2 concentration in a buffer after the addition of purified recombinant CmpA protein. A 0.5-ml aliquot of a CmpA solution (19.6 mg/ml), equilibrated by dialysis with a buffer containing 0.040 μM of dissolved CO2, was injected at time 0 into 2.5 ml of buffer containing 6 μM CO2 (170 μM C1), with (A) or without (○) 1800 units of CA/ml at 15 °C. The CO2 concentration in the buffer was measured with a mass spectrometer. In control experiments, 0.5 ml of the dialysis buffer, containing 0.040 μM of dissolved CO2, was injected in place of the protein solution (circles) at the same time (triangles) from those after injection of the protein solution (circles in A).

ml of the protein solution (19.6 mg/ml) that had been equilibrated with the buffer containing 0.040 μM CO2, the CO2 concentration decreased by 2.6 μM in 400 s, verifying the ability of the recombinant CmpA protein to bind C1. From the difference in the extent of decrease in dissolved CO2 caused by injection of the buffer and the protein solution, the concentration of the C1-binding site that bound C1 after the injection was calculated to be 45 μM. In the presence of CA, the extent of decrease in dissolved CO2 was the same as that in the absence of CA, but the decrease was faster and completed in 25 s after the injection of the protein solution (Fig. 2A). These results indicated that the binding of C1 to the protein caused temporary disequilibrium of CO2 versus HCO3 in favor of CO2, strongly suggesting that the CmpA protein binds HCO3 rather than CO2.

The time course of decrease in CO2 due to binding of C1 to CmpA, excluding the dilution effect, was calculated by subtracting the data obtained by the addition of buffer from those obtained by the addition of protein solution (Fig. 2B). From the curves, the initial rates of decrease in CO2 concentration were calculated to be 0.118 μM/s and 0.0152 μM/s in the presence and absence of CA, respectively. When 50 μM NaHCO3 was added to the buffer in the absence of CA, the initial rate of increase in CO2 concentration was 0.0180 μM/s (data not shown), which was similar to the initial rate of decrease in CO2 concentration caused by removal of 45 μM of C1 from medium due to binding to CmpA. Furthermore, a hypothetical CO2-binding protein would have been expected to cause a rapid and pronounced decline in the CO2 concentration, in the absence of CA, followed by a slow return back to the equilibrium level. This clearly did not occur. These results also support the notion that it is HCO3 that bound to CmpA.

Equilibrium Dialysis Experiments at Low C1 Concentrations—Although the equilibrium dialysis using 14C-labeled HCO3 showed specific binding of C1 to the CmpA protein, kinetic analysis of C1 binding was hardly possible by this method because of contamination by atmospheric 12CO2. We therefore performed equilibrium dialysis with no added C1, with continuous sparging of the dialysis buffer with N2 gas to keep the concentrations of C1 in the buffer low, and used mass spectrometry for quantitation of 14C in the aqueous solutions. Different final C1 concentrations were obtained by varying the rate of sparging with N2 and the volume of the dialysis buffer. The concentration of HCO3 bound to CmpA was calculated by subtracting the total C1 concentration in the dialysis buffer from that in the protein solution. The free HCO3 concentration was calculated from the total C1 concentration in the dialysis buffer and the fraction of HCO3 in total C1 at 30 °C at the pH of assay buffer (74 and 96.5% at pH 7.0 and pH 7.5, respectively). The recombinant CmpA protein was found to bind HCO3 with similar binding kinetics at pH 7.0 and pH 7.5 (Fig. 3). From the Scatchard plots (15) of the data, the dissociation constant for HCO3 was calculated to be 5.8 and 5.0 μM at pH 7.0 and pH 7.5, respectively. These values were similar to the protein concentration used, 220 μM, as calculated from the protein concentration of 10.9 mg/ml and the calculated molecular mass of 49,113 Da, suggesting that one molecule of protein carries one substrate-binding site.

Inhibition of PEPCase Activity with the Recombinant CmpA Protein—Fig. 4A compares the dependence of PEPCase activity on HCO3 concentration in assay buffer in the absence and presence of the recombinant CmpA protein. In the absence of CmpA, the maize PEPCase preparation used in this study showed a saturation kinetics with a Km (HCO3) value of 32 μM, which was similar to the previously reported value (20 μM) (16). In accordance with the ability of CmpA to bind HCO3 with high affinity, the presence of CmpA was found to competitively inhibit the PEPCase reaction. The inhibitory effect was more prominent at lower concentrations of HCO3, resulting in a sigmoidal response of the rate of PEPCase reaction on the HCO3 concentration. At a given concentration of total HCO3 in the CmpA solution, the concentration of free HCO3 was obtained as the HCO3 concentration that supported the same rate of PEPCase reaction in the absence of CmpA. The concentration of CmpA-bound HCO3 was then calculated by subtraction of the free HCO3 concentration from the total HCO3 concentration. Fig. 4B shows the relationship between the concentrations of free HCO3 and CmpA-bound HCO3 thus obtained. From the Scatchard plot (15) of the data (Fig. 4B, inset), the dissociation constant was calculated to be 5.4 μM. The calculated concentration of the bound HCO3 under saturation
was 60.5 μM. Repeated measurements using the same protein sample yielded a $K_d$ value of 5.35 ± 0.11 μM with the concentration of the binding site being 60.6 ± 2.2 μM ($n = 3$), showing the reproducibility of the method for determination of the parameters. The calculated concentration of the $\text{HCO}_3^-$ binding site was close to the protein concentration used, 61 μM, as calculated from the protein concentration of 3.0 mg/ml and the molecular mass of the protein, suggesting the presence of one substrate-binding site per one molecule of the protein. These results were essentially the same as those obtained by the equilibrium dialysis analyses using a mass spectrometer as the means of $\text{Ci}$ quantitation.

**DISCUSSION**

Bacterial ABC importers require a substrate-binding protein that has high affinity for its specific substrate (17, 18). In this study, a recombinant CmpA protein was shown to bind $\text{HCO}_3^-$ (Fig. 2), which conforms with our previous finding that the ABC transporter encoded by the $\text{cmpABCD}$ operon acts as an inducible, high affinity transporter for $\text{HCO}_3^-$ in $\text{Synechococcus}$ strain PCC 7942 (3). The dissociation constant of the $\text{HCO}_3^-$ binding site was close to the protein concentration used, 61 μM, as calculated from the protein concentration of 3.0 mg/ml and the molecular mass of the protein, suggesting the presence of one substrate-binding site per one molecule of the protein. These results were essentially the same as those obtained by the equilibrium dialysis analyses using a mass spectrometer as the means of $\text{Ci}$ quantitation.

The CmpA protein was originally found as a major protein of the cytoplasmic membrane of the cells grown under $\text{CO}_2$-limited conditions (19). It is tightly bound to the cytoplasmic membrane despite the hydrophilicity of the predicted sequence (4). In a previous study, we showed that NrtA, a paralog of CmpA, is a substrate-binding lipoprotein, which is anchored to the cytoplasmic membrane by the lipid moieties attached covalently to the N-terminal Cys residue of the mature protein (9). Maturation of the lipoproteins requires signal peptidase II for cleavage of the signal peptide from an $\text{S}$-glyceride derivative of prolipoproteins with the modified Cys residue at the signal cleavage site (20). Similar to the case in NrtA, the predicted amino acid sequence around the presumed signal cleavage site of CmpA, LKGC (9), conforms to the consensus sequence recognized by signal peptidase II, $(L/V/I)(A/S/T/G)(G/A)C$, in which one mismatch is acceptable in the first two amino acids (21). The N terminus of the mature CmpA protein, purified from the cytoplasmic membrane of low $\text{CO}_2$-grown $\text{Synechococcus}$ cells, was shown to be blocked (4). The His-tagged recombinant CmpA protein was expressed as a soluble protein in $\text{E. coli}$ (Fig. 3).

**FIG. 3. Binding of $\text{HCO}_3^-$ to the purified recombinant CmpA protein as a function of the substrate concentration.** Aliquots of the solution of purified protein (10.9 mg/ml) were dialyzed separately against various volumes of buffer having pH of 7.0 (A) or 7.5 (B), with continuous sparging with $\text{N}_2$ at various rates at 30 °C for 18 h. After the dialysis, concentrations of total $\text{Ci}$ and free $\text{CO}_2$ in the protein solutions and the dialysis buffer were measured with mass spectrometer. The concentration of the bound $\text{HCO}_3^-$ [$\text{HCO}_3^-$ bound] was plotted against that of free $\text{HCO}_3^-$, Insets, Scatchard plots of the data.

**FIG. 4. Inhibition of PEPCase activity with the recombinant CmpA protein.** A, PEPCase activity in the presence (○) and absence (●) of purified CmpA protein (3.0 mg/ml). PEPCase activity was assayed at 30 °C in a buffer containing 50 mM TES-NaOH, pH 7.5, 5 mM $\text{MgCl}_2$, 2 mM dithiothreitol, 0.8 mM NADH, 6 mM phosphoenolpyruvate, and 0.5 mM Na$\text{HCO}_3$. $\text{HCO}_3^-$ concentration was calculated from the decrease in NADH concentration (see “Experimental Procedures”). PEPCase activity was plotted against the concentration of $\text{HCO}_3^-$, B, binding kinetics of bicarbonate to a recombinant CmpA. At a given [$\text{HCO}_3^-$] total in the CmpA solution (A), [$\text{HCO}_3^-$] bound was evaluated as the $\text{HCO}_3^-$ concentration that provided the same rate of PEPCase reaction in the absence of CmpA. [$\text{HCO}_3^-$] bound was then calculated by subtraction of [$\text{HCO}_3^-$] free from [$\text{HCO}_3^-$] total and plotted against [$\text{HCO}_3^-$] free, Inset, Scatchard plot of the data.
paralog of CmpA forms a gene cluster with the genes, which encode membrane components of an ABC transporter orthologs of CmpA (Slr0040 of Synechocystis (27); NasF of Cyanobase) of 87,787–86,411 on the c374 segment of genomic DNA sequence; 6803 (29), the protein encoded by an open reading frame (bases Synechocystis to together with the orthologs of NrtA (NrtA of Anabaena sp. strain PCC 7120) (22). The CmpA protein, which is 28 and 26% identical to CmpA and NrtA, respectively, is hence supposed to be the substrate-binding protein of the putative cyanate transporter. Tam and Saier (18) previously identified eight groups of substrate-binding proteins, which are distinct in primary amino acid sequences and the nature of the substrate. The CmpA, NrtA, and CynA proteins appear to constitute the ninth distinct group of substrate-binding proteins, which are involved in binding of monoanions, together with the orthologs of NrtA (NrtA of Anabaena sp. strain PCC 7120 (23, 24), Synechocystis sp. strain PCC 6803 (25), Phormidium laminosum (26), and Plectonema boryanum (27); NasF of Klebsiella pneumoniae (28)) and the putative orthologs of CmpA (Slr0040 of Synechocystis sp. strain PCC 6803 (29), the protein encoded by an open reading frame (bases 87,787–86,411 on the c374 segment of genomic DNA sequence; Cyanobase) of Anabaena sp. strain PCC 7120). It should be also noted that there is another class of CmpA/NrtA homologs in cyanobacteria. These are the C-terminal domain of CmpC and that of NrtC. CmpC and NrtC are each one of the two ATP-binding subunits of the bicarbonate- and nitrate/nitrite transporters, respectively, and are composed of two distinct domains. Whereas their N-terminal domains are strongly similar to the ATP-binding subunits of other ABC transporters and to each other, their C-terminal domains are 30% identical to CmpA, NrtA, and to each other. The C-terminal domain of NrtC is required not for nitrate/nitrite transport but for ammonium-promoted inhibition of the transport (30), indicating that it is a regulatory domain of the transporter. The similarity of the regulatory domain of NrtC to CmpA/NrtA and the capacity of CmpA/NrtA for substrate binding suggest that the regulation of nitrate/nitrite transport might involve bind-

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