Carbonyl Sulfide: An Inhibitor of Inorganic Carbon Transport in Cyanobacteria¹

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ABSTRACT

Cells of a high CO₂-requiring mutant (E₁) and wild type of Synechococcus PCC7942 were incubated with COS in the light, then suspended in COS-free medium and their CO₂ exchange was measured using an open gas-analysis system under the conditions where photosynthetic CO₂ fixation is inhibited. When the suspension of cells untreated with COS was illuminated, the rate of CO₂ uptake was high and addition of carbonic anhydrase during illumination released a large amount of CO₂ from the medium into the gas phase. The COS treatment in the light markedly reduced the rate of CO₂ uptake by the cells and the amount of CO₂ released by carbonic anhydrase. Incubation of cells with COS in the dark had no effect on the CO₂-exchange profile. The COS concentration required for 50% inhibition of CO₂ uptake was about 25 micromolar when the concentration of inorganic carbon (Ci) in the medium was 60 micromolar; higher Ci concentrations reduced the inhibitory effect of COS. Measurement of Ci uptake in E₁ cells by a silicone oil centrifugation method also indicated marked reduction of the activities of ¹⁴CO₂ and H¹⁴CO₂⁻ uptake in the cells treated with COS in the light. The results demonstrated that COS is a potent inhibitor of Ci transport.

Cyanobacteria possess a Ci-translocating system which is activated and energized by light (4, 5, 9, 11, 12). The activation requires PSI and energization can occur only by PSI (5, 12, 15, 16). Both CO₂ and HCO₃⁻ are transported into the cells and HCO₃⁻ appears to be the species which arrives at the inner membrane surface, regardless of the species supplied (20). The nature of the Ci-translocating system is poorly understood. The activation or energization is inhibited by a number of inhibitors of electron transport or ATP synthesis of photosynthesis (5, 16). However, inhibitors which interact directly with the Ci-translocating system or 'Ci-transporter' have not been reported. Such inhibitors would be useful in elucidating the nature of the Ci-translocating system.

Carbonyl sulfide is a competitive inhibitor of Rubisco with respect to CO₂ and is an alternative substrate of ribulose-1,5-bisphosphate (6, 8). In Synechococcus PCC 7942, CO₂ is the species preferentially taken up by the cells into the internal Ci pool and it was expected that COS could be a competitive inhibitor of the Ci-translocating system. We examined in this study the effect of COS on the Ci-translocating activity of a high CO₂-requiring mutant, E₁ (18) and the wild type of Synechococcus PCC7942. Three methods were used to evaluate the effect of COS on Ci transport: i.e. gas exchange measurements in an open system and by a mass spectrometer, and measurements of ¹⁴CO₂ and H¹⁴CO₂⁻ uptake by the silicone oil-centrifugation method. We report here the effect of COS on CO₂ uptake by E₁ and wild-type cells and provide evidence that COS is a potent inhibitor of Ci transport.

MATERIALS AND METHODS

Treatment of Cyanobacterial Cells by COS. Cells of wild-type and E₁ mutant of Synechococcus PCC7942 (Anacystis nidulans R₂) were grown under 3% CO₂ in air as described previously (13). Continuous illumination was provided by tungsten lamps (120 μmol PAR/m²-s). At a late logarithmic phase of growth the cultures were aerated in air (0.04% CO₂) for 20 h under the same conditions. Cells were harvested by centrifugation and suspended in 40 mm HEPES-NaOH (pH 7.0) containing 20 mm NaCl at a concentration of about 5 μgchl/ml. Aliquots (25 ml each) of the cell suspension were placed in screw-capped transparent tubes, and after addition of COS (in water), the suspension was illuminated at 30°C with white light (1.0 mmol PAR/m²-s) for 10 min. Cells were then collected by centrifugation and used for the measurements of Ci-transporting activity by an open gas-analysis system and by a silicone oil-centrifugation method. Before the measurements, COS-free air was bubbled through the suspension of COS-treated cells to remove residual COS from the medium.

Synthesis and Analysis of COS. COS was obtained by hydrolysis of KCNS with H₂SO₄ as described by Stock and Kuss (18). COS was bubbled successively through 30% KOH in water, so as to minimize contamination by CO₂, and collected in a test tube by displacing water. The test tube containing COS and a few ml of water was then vigorously shaken to solubilize COS in water. The concentration of COS in water was determined after COS was converted to KSCOOC₂H₅ in an alcoholic potassium hydroxide solution; the solution was made slightly acidic with acetic acid and was titrated with iodine (2).

Gas-Exchange Measurements. Two gas-analysis systems were used. One is an open system described previously (14) which records the rate of gas exchange directly, and the other is a mass-spectrometer (TE-150, ANELVA, Tokyo) with a reaction vessel essentially the same as that described by Badger and Andrews (1). For the measurements in the open system, cells were suspended in 25 ml of 40 mm HEPES-NaOH (pH 7.0) containing 20 mm NaCl at a Chl concentration of about 5 μg/ml and placed in a reaction vessel kept at 30°C. Nitrogen containing 400 μL

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² Abbreviations: Ci, inorganic carbon; CA, carbamic anhydrase; IAc, iodoacetamide; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; DMQ, 2,5-dimethyl-p-benzoquinone; PNDa, p-nitrosodimethylaniline.
CO₂/L and 2% O₂ was bubbled into the cell suspension and CO₂ concentration in the exchanged gas was analyzed by an IR CO₂ analyzer (model ZAP, Fuji Electric Co., Tokyo). When O₂ evolution was measured, nitrogen gas was bubbled into the cell suspension and O₂ concentration in the exchanged gas was analyzed by a trace oxygen analyzer (model 316; Teledyne Analytical Instrument Co., City of Industry, CA).

In the measurements by the mass-spectrometer, cells (untreated with COS) were suspended in 40 mM Hepes-NaOH (pH 8.0) containing 20 mM NaCl, placed in the reaction vessel and then COS (in water) was added to the cell suspension. Gases in the suspension were led into the mass-spectrometer through a silicon membrane (silastic sheeting 500-1; Dow Corning, Midland, MI), and concentrations of CO₂ and COS were monitored at the Midland mass numbers of 44 and 60, respectively.

**Measurements of CO₂ and HCO₃⁻ uptake.** The uptake of CO₂ and HCO₃⁻ into the intracellular Ci pool was measured at 30°C by the silicone oil-filtering centrifugation method (4, 20). Cells were suspended in 40 mM Hepes-NaOH (pH 8.0) containing 20 mM NaCl. The reaction was initiated by adding HCO₃⁻ or H₂CO₃ (final concentration of 16.7 and 190 µM, respectively; 55 µCi/µmol) in the light and terminated by centrifugation through a silicone layer (SH550:SH556, 32:68V/V; Toray Silicon Co., Tokyo).

**Determination of ATP.** Approximately 1 mL of cell suspension was drawn rapidly from the reaction vessel into a 1.5 ml syringe containing 125 µL of 70% perchloric acid. The extract was neutralized by adding 5 mM KOH/1 mM trithanolamine and ATP in the neutralized extract was determined by the firefly luciferase procedure as described by Lilley et al. (7).

**RESULTS**

**CO₂ Exchange of COS-Treated Cells.** Cells of E₁ mutant were illuminated for 10 min in the presence or absence of COS and were suspended in COS-free medium (40 mM Hepes-NaOH [pH 7.0] containing 20 mM NaCl), and then their CO₂ exchange rates were measured in an open gas-analysis system. The CO₂ exchange profile of the cells illuminated in the absence of COS (curve A in Fig. 1) is essentially the same as that previously reported (14). This mutant does not perform CO₂-dependent O₂ evolution at air levels of CO₂, although it has the ability to accumulate C₃ internally (10, 17). Thus, the uptake of CO₂ by the mutant at these low CO₂ conditions is independent of photosynthetic CO₂ fixation and represents the activity of the C₃-transporting system. The evolution of CO₂ from the medium into the gas phase on addition of CA in the light indicates that a portion of CA taken up by the mutant is eroded from the cells and partly stays in the medium as HCO₃⁻, producing nonequilibrium conditions between CO₂ and HCO₃⁻ in the medium with the concentration of HCO₃⁻ being higher than the expected under equilibrium conditions (13, 14). The amount of CO₂ evolved after darkening in the presence of CA in the medium represents the size of the intracellular Ci pool (13).

The maximal rate of CO₂ uptake (M, Fig. 1) in the cells which had been illuminated in the presence of COS (COS-treated cells, curve B) was much lower than that in the cells preilluminated in the absence of COS (untreated cells; curve A). Thus, COS treatment strongly inhibited the C₃-transporting activity. Treatment of the cells with COS in the dark had no effect on M (data not shown), indicating that the inhibition of C₃ transport by COS requires light. The amount of CO₂ evolved in the light by addition of CA was also much lower in the COS-treated cells than in the untreated cells, showing that the production of nonequilibrium conditions between CO₂ and HCO₃⁻ in the medium is not marked in the COS-treated cells. In contrast, there was not much difference between the COS-treated and untreated cells in the amount of CO₂ evolved after darkening. Thus, COS-treatment did not have significant effect on the size of the intracellular Ci pool in the light in this mutant.

Experiments with the wild type cells (measured in the presence of IAc, an inhibitor of CO₂ fixation) gave the essentially the same result as that obtained with E₁ mutant, except that the amount of CO₂ evolved after darkening in the presence of CA was much less in the COS-treated cells (Fig. 2). Probably, the rate of extrusion of intracellular Ci is high in the wild-type cells and the suppression of the Ci influx by the COS treatment made it impossible to keep the intracellular Ci pool at high level. The activity of O₂ evolution dependent on photosynthetic CO₂ fixation (measured in the absence of IAc) was completely abolished in the COS-treated cells of the wild type, even under high concentrations of CO₂ (e.g. 1%; data not shown). Probably, COS inactivated Rubisco irreversibly or the formation of 3-phosphoglycerolate (8) prevented the further reactions of the C₃-pathway.

CO₂ reacts slowly with water to produce CO₂ and H₂S. Since H₂S (or HS⁻) is a potent inhibitor of photosynthesis, the observed inhibition of Ci transport in the COS-treated cells could be a result of irreversible inhibition of electron transport or ATP generating reactions by H₂S. We have measured the activity of O₂ evolution in the COS-treated E₁ cells with DMQ or PND such as electron acceptors and ATP generation in the absence of electron acceptors. DMQ and PND accept electrons from plastoquinone (19) and ferredoxin (3), respectively. The results shown in Table I indicate that the ATP level and the activity of O₂ evolution in the COS-treated cells were not much different from those in the untreated cells. Thus, the inhibition of Ci transport by COS is not due to inhibition of energy generating reactions.

**Effect of COS and NaHCO₃ Concentrations.** E₁ cells were preilluminated for 10 min in the presence of various concentrations of COS under the conditions where the Ci concentration at the start of preillumination was 63 µM and then their M values were...
had COS, uM. preillumination, and/or were incubated treatments.

1) (Fig. 1) determined in the COS-free medium. Figure 3, showing the M values as a function of COS concentration during the preillumination, indicates that the 50% inhibition occurs at a concentration around 25 $\mu$M. COS had no effect when cells were incubated in darkness even at a high concentration of 180 $\mu$M.

The extent of inhibition by COS was strongly affected by the concentration of C$_1$ present in the medium during the COS treatment. The M values determined with E$_1$ cells preilluminated for 10 min in the presence of 60 $\mu$M COS and various concentrations of C$_1$ are shown in Figure 4 as a function of C$_1$ concentration. COS had strong inhibitory effect when C$_1$ concentration was low and became less inhibitory as the C$_1$ concentration increased. The results clearly showed that COS inhibition is prevented by CO$_2$ and/or HCO$_3^-$.

Inhibition of CO$_2$ and HCO$_3^-$ uptake by COS. The activities of $^{14}$CO$_2$ and H$^{14}$CO$_3^-$ uptake into the intracellular C$_1$ pool were measured with E$_1$ cells by a silicone oil-centrifugation method.

![Fig. 2](image-url) Changes in CO$_2$ concentration in the gas phase of a suspension of wild-type cells treated with 3 mm IAc upon switching the light on and off and by addition of CA in the light. Cells were preilluminated in the absence (curve A) or presence (B) of COS. IAc was not added during the preillumination.

![Fig. 3](image-url) Maximal rate of CO$_2$ uptake (M, Fig. 1) in E$_1$ cells as a function of the concentrations of COS in the medium during the preillumination. The C$_1$ concentration at the start of preillumination was 70 $\mu$M. M is expressed in percent of the control value (135 $\mu$mol/mg Chl h).

![Fig. 4](image-url) Maximal rate of CO$_2$ uptake (M) in E$_1$ cells as a function of the concentrations of C$_1$ in the medium during the COS treatment in the light. The COS concentration was 60 $\mu$M. M is expressed in percent of the control value (146 $\mu$mol/mg Chl h).

Curves A and C in Figure 5 show the time courses of accumulation of acid-labile $^{14}$C within the untreated cells of E$_1$ mutant in the light after addition of $^{14}$CO$_2$ and H$^{14}$CO$_3^-$, respectively, into the cell suspension. Cells took up CO$_2$ much faster than HCO$_3^-$. The rate of HCO$_3^-$ uptake was very low when the HCO$_3^-$ concentration was equal to the CO$_2$ concentration, i.e. 16.7 $\mu$M (data not shown), and was only one-fifth the rate of CO$_2$ uptake even at the HCO$_3^-$ concentration of 190 $\mu$M (curve C, Fig. 5). Thus, CO$_2$ is the species preferentially taken up by E$_1$ cells.

The uptake of CO$_2$ was strongly suppressed in the COS-treated cells (curve B, Fig. 5). The suppression was irreversible, suggesting that COS strongly bound to the active site of the CO$_2$-transporting system. The uptake of HCO$_3^-$ was also suppressed in the COS-treated cells (curve D). The results suggested that the CO$_2$-transporting system sensitive to COS is also functioning in transporting HCO$_3^-$. COS Uptake. The uptake of CO$_2$ and COS was measured with E$_1$ cells using a mass spectrometer. Unlike the measurements in the open system or by the silicone oil-centrifugation method, the uptake of CO$_2$ was measured in the presence of COS with...
Fig. 5. Changes in intracellular C\textsubscript{i} pool (acid-labile \textsuperscript{14}C) of E\textsubscript{i} cells with incubation time in the light, measured by the silicone oil-centrifugation method. A, C: Cells preilluminated for 10 min in the absence of COS; B, D: cells preilluminated in the presence of 190 \textmu M COS. The C\textsubscript{i} concentration at the start of preillumination was 70 \textmu M. A: \textsuperscript{14}CO\textsubscript{2} (16.7 \textmu M, final concentration) was added; C, D: H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{−} (190 \textmu M) was added.

Fig. 6. Changes in concentrations of CO\textsubscript{2} (curve a, b, c) and COS (b') in the suspension of E\textsubscript{i} cells upon switching the light on and off. The cell suspension contained 530 \textmu M C\textsubscript{i} plus 0 (curve a), 21 (b and b'), and 209 (c) \textmu M COS, respectively. CO\textsubscript{2} and COS were monitored at the mass numbers of 44 and 60, respectively. The CO\textsubscript{2} concentration in the cell suspension in the dark was 11 \textmu M.

untreated cells. The concentration of COS in the medium was also monitored simultaneously; it was not possible to obtain reliable results when the COS concentration was 209 \textmu M. Figure 6 shows the changes in CO\textsubscript{2} and COS concentrations in the cell suspension upon switching the light on and off. When the cell suspension was illuminated in the absence of COS (curve a), the CO\textsubscript{2} concentration in the medium decreased rapidly to reach a steady state in 90 s. Turning the light off resulted in an increase of the CO\textsubscript{2} concentration in the medium to the original level. Since E\textsubscript{i} cells do not fix CO\textsubscript{2} under the CO\textsubscript{2} concentration used in these measurements, the decrease in CO\textsubscript{2} concentration in the light is ascribed to the uptake of CO\textsubscript{2} into the intracellular C\textsubscript{i} pool and also to the production of nonequilibrium conditions between CO\textsubscript{2} and HCO\textsubscript{3}− in the medium with the concentration of HCO\textsubscript{3}− being higher than that expected under equilibrium conditions (cf. Fig. 1). The initial rate of decrease in CO\textsubscript{2} concentration was 60% of the control value when the cells were illuminated in the presence of 21 \textmu M COS (curve b) and was 10% at 209 \textmu M COS (curve c). Thus, the presence of COS in the medium strongly inhibits the C\textsubscript{i}-transporting activity. Simultaneous measurement of COS uptake under the conditions where the initial COS concentration was 21 \textmu M (curve b') indicated that COS was slowly taken up by the cells in the light. There was no efflux of COS in the dark after turning off the light. Thus, COS appears to be incorporated into organic substances in the light.

DISCUSSION

The present study demonstrated that COS is a potent inhibitor of C\textsubscript{i} transport in cyanobacteria. The activity of CO\textsubscript{2} uptake into the intracellular C\textsubscript{i} pool was suppressed in cells preilluminated in the presence of COS, even after COS was completely removed from the medium (Figs. 1–5). Thus, the inhibition was irreversible. Incubation of cells with COS in the dark had no effect on the C\textsubscript{i}-transporting activity, which indicated that the inhibition required light. These results suggested that COS reacted with the active site of the C\textsubscript{i}-transporting system in the light and this led to the inhibition of C\textsubscript{i} transport. Measurement of COS uptake by a mass spectrometer showed that COS was slowly taken up by the cells in the light but was not extruded from the cells after darkening (Fig. 6). Evidently, COS is not taken up into the intracellular C\textsubscript{i} pool as a substitute for CO\textsubscript{2} and appears to be incorporated into organic substances.

Lorimer and Pierce (8) recently reported that COS reacts with ribulose 1,5-bisphosphate to produce 3-phosphoglycerate and 3-phospho-1-thioglycerate when Rubisco activated with CO\textsubscript{2} was used. They also showed that COS displaced the activator carbamate in Rubisco but the resultant monothiocarbamylated enzyme was not catalytically competent. The inhibition of C\textsubscript{i} transport by COS cannot be the result of inactivation of Rubisco or inhibition of CO\textsubscript{2} fixation, since C\textsubscript{i} transport is insensitive to inhibitors of CO\textsubscript{2} fixation (15, 16). It appears possible that the formation of a carbamate is involved in the C\textsubscript{i}-transporting mechanism and displacement of the carbamate with COS leads to the inhibition of C\textsubscript{i} transport. We are now trying to label the cells with \textsuperscript{14}COS in an attempt to find a protein which reacts with COS in the light.

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