Glutamine and Glutamate Transport in *Cyanophora paradoxa*

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Abstract

The present investigation showed that isolated cyanelles from *Cyanophora paradoxa* selectively enriched glutamine from the external medium, whereas glutamate poorly penetrated into these organelles. Glutamine uptake proceeded in two phases, presumably involving a low and a high affinity system. The uptake of glutamine was significantly enhanced by 2-oxoglutarate and light. Inhibitor experiments indicated that glutamine and 2-oxoglutarate were converted to glutamate by a ferredoxin-dependent glutamate synthase (GOGAT) reaction inside the cyanelles, and the glutamate formed at best slowly left these organelles. Such results were obtained independently of each other by measuring either the ^{14}C-glutamine uptake or the 2-oxoglutarate and glutamine-dependent O_2 evolution. Glutamine is suggested to be the N-compound which is supplied to the eukaryotic host. Glutamine could be exported jointly with 2-oxoglutarate, possibly employing a common carrier. Cyanelles have apparently evolved glutamine (and oxoglutarate) carrier(s) with properties not yet described for any other organism.

Key words

Glutamine carrier, glutamate transport, oxoglutarate transporter, glutamate synthase, cyanelles, *Cyanophora paradoxa*,

Abbreviations and Symbols

chl.:	chlorophyll
DCMU:	N′-(3,4-dichlorophenyl) N,N-dimethylurea
FCCP:	carbonylcyanide-*p*-trifluoro-methoxyphenyl-hydrazone
GS:	glutamine synthetase
GOGAT:	glutamine: oxoglutarate amido-transferase = glutamate synthase
MSX:	methionine sulfoximine
2-OG:	oxoglutarate
PAPS:	3′-phosphoadenosine-5′-phosphosulfate

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Introduction

Cyanelles are tightly incorporated into the cell-metabolism of *Cyanophora paradoxa* like chloroplasts in higher plants. They perform photosynthetic CO_2-fixation by the conventional reductive pentose phosphate cycle, and the results of the accompanying paper (Schlichting and Bothe, 1993) showed that carbon is allocated mainly by a phosphate translocator to the eukaryotic host. *Cyanophora paradoxa* is a C, N and S autotroph, therefore, cyanelles might also serve in reducing sulfate and nitrate. Sulfate reduction has not been investigated as yet, but the demonstration of a PAPS-dependent sulfotransferase in cyanelles (Schmidt and Christen, 1979) could indicate that sulfate reduction takes place in them. A previous study (Floener et al., 1982) showed that nitrate reductase is located in the eukaryotic host and that nitrite reductase resides in cyanelles as in higher plants. The previous investigation also showed that cyanelles contain glutamine synthetase (GS) and glutamate synthase (GOGAT) which were also found in the eukaryotic host in contrast to nitrite reductase. The N-compounds which are allocated to the eukaryotic host have not been determined as yet. The present investigation, therefore, characterizes the transfer of glutamine and glutamate to cyanelles by the silicone oil centrifugation technique.

Materials and Methods

The culture conditions for *Cyanophora paradoxa* and the isolation of cyanelles have been described in the accompanying (Schlichting and Bothe, 1993) and preceding papers (Schlichting et al., 1990).

The uptake of glutamine and glutamate into cyanelles was determined by the silicone oil filtering centrifugation technique (Schlichting et al., 1990; Schlichting and Bothe, 1993). The reaction mixture on top of the silicone oil layer contained 0.5–1.0 mM glutamine or glutamate labeled with 3.7–7.4 kBq [U-^{14}C] glutamine or glutamate, respectively, 37 kBq [^{3}H]-water and cyanelles in the isolation buffer with 330 mM sucrose, 50 mM Tricine/KOH pH 8.0, 2 mM EDTA and 1 mM $MgCl_2$ containing 10–13 µg chl. For the determination of the sucrose-impermeable space, the reaction mixture contained 7.4 kBq [U-^{14}C]-sucrose, 37 kBq [^{3}H]-water and cyanelles with 10–13 µg chl. The incubation was performed at room temperature in the light (12 000 lm m^{-2}) or dark. The reaction was terminated by centrifuging the cyanelles through the silicone oil layer into the $HClO_4$ fraction within 5 s using a Beckman microfuge B.

The 2-OG and glutamine-dependent O_2 uptake was measured amperometrically using a conventional Clark-type electrode. The electrode chamber contained, in a final vol of 1.6 ml: *L*-glutamine 1 mM, 2-oxoglutarate 1 mM, cyanelles with 30 μg chl. and inhibitors as indicated in Table **2**. The experiment was performed at 25 °C and 12 000 lm m^{-2} at the surface of the chamber.

Results

The uptake of glutamine and glutamate into cyanelles was investigated by the silicone oil filtering technique exactly as described in the accompanying (Schlichting and Bothe, 1993) and previous (Schlichting et al., 1990) papers. When the experiments were performed at room temperature, pH 8.0 and an external substrate concentration of 1 mM, cyanelles enriched glutamine by 1.7 fold within 10 min (Fig. **1**). The uptake rate was determined from the linear onset of the curve within the first min to be about 250 nmol h^{-1} mg chl^{-1}. In contrast, glutamate only poorly penetrated into cyanelles with an enrichment factor of 0.5 and with a low initial rate of about 15 nmol h^{-1} mg chl^{-1}.

The determination of the uptake rates in dependence of the amount of glutamine or glutamate offered externally showed that glutamine uptake proceeded in two phases (Fig. **2**). In the lower concentration range (0.05–4 mM), the apparent K_m and V_{max} were 0.5 mM and 360 nmol h^{-1} mg chl^{-1}, respectively, for glutamine determined from the Eadie-Hofstee plot (Fig. **2**, insert). In the higher concentration range (>4 mM), glutamine could have penetrated into cyanelles by diffusion or by a second, low affinity carrier. This could not be resolved accurately, since the reaction did not show saturation even at 10 mM glutamine (Fig. **2**). The kinetic parameters for glutamate were K_m = 0.1 mM and V_{max} = 29.6 nmol h^{-1} mg chl^{-1}. Glutamate uptake seemingly proceeded in two phases (Fig. **2 a**, insert), but the low uptake rates for this amino acid did not allow to resolve this distinctly.

Glutamine uptake rates increased almost linearly with increasing pH in the range 5.5–8.0. In contrast, the difference value between the water space and sucrose space (assumed to be equivalent to the internal volume of cyanelles, see Schlichting et al., 1990) did not significantly vary between pH 6.0 and 8.0 (not shown). The strong pH-dependence of glutamine uptake probably meant that this amino acid crossed the cytoplasmic membrane as a zwitterion.

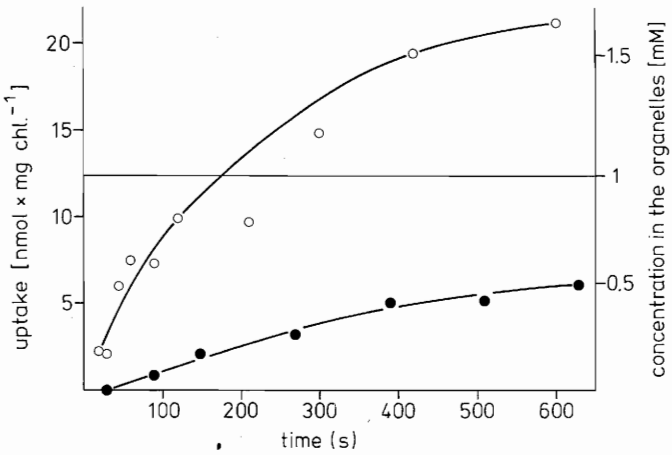

Fig. 1 The uptake of glutamine and glutamate into isolated cyanelles determined by the silicone oil filtering technique.
The experiment was performed at pH 8.0, room temperature and daylight. The samples above the silicone oil layer contained in a final vol of 200 μl **a**) for glutamine uptake: glutamine, 1 mM labeled with 3.7 kBq [U-^{14}C] glutamine, 37 kBq [^{3}H]-H_2O, cyanelles with 13 μg chl. **b**) for glutamate uptake: glutamate 1 mM labeled with 7.4 kBq [U-^{14}C] glutamate, 37 kBq [^{3}H]-H_2O, cyanelles with 10 μg chl., **c**) for the determination of the sucrose impermeable space: 0.33 mM sucrose labeled with 7.4 kBq [^{3}H]-H_2O, cyanelles with 13 μg chl. The reaction was terminated by centrifuging the cyanelles through the silicone oil layer into the 1.2 M $HClO_4$ fraction within 5 s.
O—O glutamine uptake
●—● glutamate uptake
Straight horizontal line: indicates the external concentration (= 1 mM).

The uptake of glutamine into cyanelles was only slightly enhanced by light (enrichment factor = 2.2 for the assays performed in light and = 1.7 in dark controls after 10 min, when 1 mM glutamine was offered). The reaction was not affected by DCMU but was severely inhibited by FCCP (75 % inhibition by 8 μM FCCP) (Table **1**).

Table 1 Characterization of the 2-oxoglutarate- and light-stimulated uptake of glutamine into cyanelles.

assay condition	uptake rate (nmol mg $chl.^{-1}$ h^{-1}) dark		light	
A. glutamine (0.5 mM) alone	132	(100 %)	143	(108 %)
+ methionine sulfoximine (2.5 mM)	111	(84 %)		n.d.
+ glutamate (2.5 mM)	0	(0 %)		n.d.
+ DCMU (5 μM)		n.d.	140	(106 %)
+ FCCP (8 μM)		n.d.	33	(25 %)
B. glutamine (0.5 mM) + oxoglutarate (2.5 mM)	317	(240 %)	495	(375 %)
+ azaserine (2.5)	38	(29 %)	39	(30 %)
+ DCMU (5 μM)		n.d.	152	(115 %)
+ FCCP (8 μM)		n.d.	310	(235 %)

The reaction was performed under standard conditions with a reaction vol of 200 μl containing glutamine (0.5 mM) labeled with 3.4 kBq [U-^{14}C]-glutamine, 37 kBq [^{3}H]-water, substrate analogues and inhibitors as indicated and cyanelles with 14 μg chl. The light intensitiy was 12,000 lm m^{-2} in the illuminated samples. The uptake was followed over 5 min and the rates were determined from the initial onset from 30 s to 2 min. n.d. = not determined.

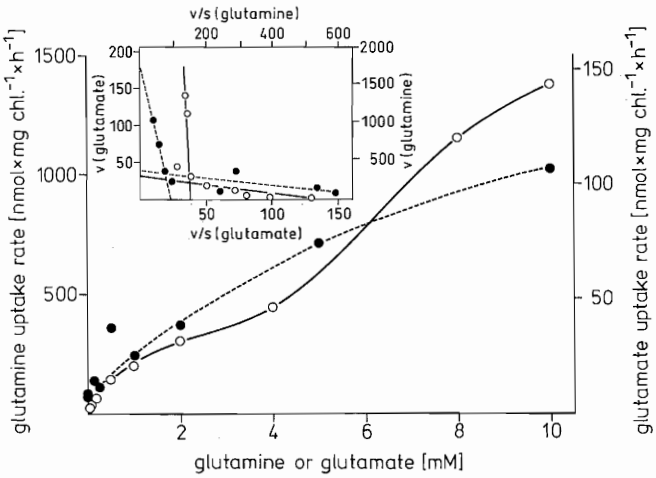

Fig. 2 Concentration-dependence of the uptake of glutamine and glutamate into cyanelles.
The assay was performed at 6 °C in the dark with 15 μg chl. in the reaction mixture. Otherwise standard conditions were employed (see legend of Fig. **1** and Table **1**). Uptakes were followed over 5 min and rates were determined from the linear onsets of the curves.
○—○ glutamine uptake
●- - -● glutamate uptake
Insert: Eadie-Hofstee plot for the determination of the kinetic parameters (for glutamine in the low concentration range).

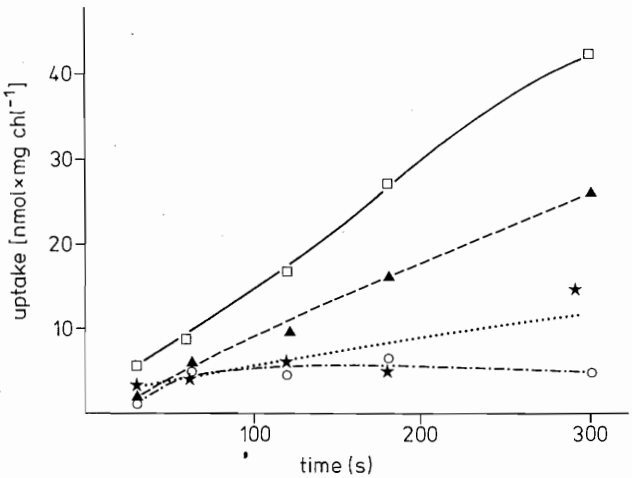

Fig. 4 Effect of inhibitors on oxoglutarate- and light-dependent glutamine uptake into cyanelles.
Isolated cyanelles (13 μg chl. in the 200 μl assay mixture) were preincubated in the light for 3 min prior to the addition of radioactively labeled glutamine (0.5 mM), oxoglutarate (2.5 mM) and inhibitors. Light intensity 12 000 lm m^{-2}.
□—□ control
▲- - -▲ + FCCP (8 μM)
★- - -★ + DCMU (5μM)
○·····○ + azaserine (2.5 mM)

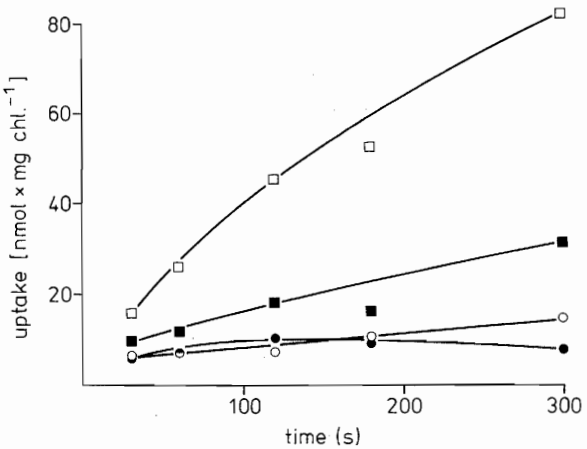

Fig. 3 Stimulation of the 2-oxoglutarate-dependent uptake of glutamine into cyanelles by light.
The reaction mixture contained 0.5 mM glutamine labeled with 3.4 kBq [U-^{14}C]-glutamine, 37 kBq [^{3}H]-H_2O, 6.7 μg chl. Oxoglutarate was 2.5 mM. Incubations were terminated after the times indicated in the abscissa.
●—● glutamine alone, dark
○—○ glutamine alone, light (12 000 lm m^{-2})
■—■ glutamine + oxoglutarate, dark
□—□ glutamine + oxoglutarate, light

The glutamine transport into cyanelles was poorly blocked by MSX and completely by glutamate, whereas the addition of 2-OG, unexpectedly, stimulated glutamine uptake about 2.4 fold (Table **1**).

The rate of the 2-OG-stimulated glutamine uptake was further enhanced when the assays were performed in light (Fig. **3**, Table **1**). Experiments with inhibitors (Fig. **4**, Table **1**) showed that this light and 2-OG-stimulated glutamine uptake was due to the glutamate synthase (GOGAT) reaction inside the cyanelles. Glutamine uptake was strongly reduced by azaserine, an inhibitor of this amidotransferase. DCMU which affects photosynthetic electron transport also blocked light and 2-OG-dependent glutamine uptake severely (Fig. **4**, Table **1**). Glutamate synthase had been demonstrated to occur in both cyanelles and the eukaryotic host (Floener et al., 1982), and it was apparently dependent on photosynthetically-generated reduced ferredoxin in cyanelles as in chloroplasts. Thus glutamine and 2-OG penetrated the cytoplasmic membrane of cyanelles and were converted to glutamate in a ferredoxin-dependent reaction in the cyanelles. As glutamate only slowly passed the cyanelle membrane, the radioactive label from the external glutamine enriched as glutamate inside the cyanelles. The light and 2-OG stimulated glutamine uptake was not affected by MSX (not shown) but to some extent by FCCP (Fig. **4**, Table **1**). The latter could indicate that the uptakes of glutamine and/or of 2-OG into cyanelles were energy-dependent.

In contrast, glutamate uptake was only slightly stimulated by light (from 15 to 19 nmol mg chl^{-1} h^{-1}) and was virtually unaffected by MSX, FCCP, DCMU and, remarkably, it was also unaffected by NH_4Cl (not shown).

As the formation of glutamate from glutamine and 2-OG required ferredoxin reduced photosynthetically, the reaction in the cyanelles could be followed also by measuring the photosynthetic O_2 production amperometrically (Table **2**). When no cofactors were added

Table 2 Demonstration of the oxoglutarate- and light-stimulated uptake of glutamine into cyanelles by the photosynthetic O_2 production.

assay condition	measured activity	O_2 production rate*
1. dark control	0.0	
2. assay in the light	−53.0	
3. + 2-OG	−35.4	17.6
4. + 2-OG + NH_4Cl	−35.4	17.6
5. + glutamine	−53.0	0.0
6. + glutamate	−53.0	0.0
7. + glutamate + NH_4Cl	−53.0	0.0
8. + 2-OG + glutamine	− 8.8	44.6
9. + 2-OG + glutamine + malate	− 4.2	49.2
10. + malate (added first) + glutamine + 2-OG	− 5.6	47.4
11. + malate	−43.8	9.2
12. + 2-OG + glutamine + DCMU (10 μM)	−43.7	9.3
13. + 2-OG + glutamine + azaserine** (6 mM)	−44.1	8.9

* Rates are given in nmol mg chl.$^{-1}$ min^{-1}. The O_2 production activity was determined by subtracting the unspecific, Mehler reaction-type O_2 uptake in the light of 53.0 nmol mg chl.$^{-1}$ min^{-1} (see text). The concentration of substances added were 1 mM each, unless indicated otherwise. The O_2 consumption activity was determined with a conventional Clark-type electrode. The assay conditions are described under Materials and Methods.
** The activity was determined after incubating the cyanelles with glutamine, 2-OG and azaserine for 1.5 min.

to the electrode vessel, illuminated isolated cyanelles showed a Mehler-type O_2 uptake. This was also observed with thylakoid preparations from the cyanobacterium *Anacystis nidulans* (Bothe, 1968) which is likely to be due to phycobilins, pterins and/or other compounds from this cyanobacterium serving as electron acceptors in these assays. The addition of glutamine alone to cyanelles did not result in O_2 formation (Table **2**), 2-OG, however, caused O_2 production. This activity was only slightly enhanced by NH_4Cl but strongly when glutamine and 2-OG were added simultaneously. The glutamine, 2-OG and light-dependent reaction was blocked by DCMU and azaserine. Thus the results obtained with the electrode and from the ^{14}C-glutamine uptake studies matched with each other. The GOGAT reaction inside the cyanelles must have been responsible for O_2 production and enhanced ^{14}C-glutamine uptake in the presence of 2-OG.

Discussion

The information on the uptake of glutamate and glutamine in cyanobacteria is scant. The reports indicate that the apparent K_m is lower for glutamine than that for glutamate, but the V_{max} for the transport of both amino acids are essentially the same (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1988; Rowell et al., 1977). Cyanobacteria may employ a low and a high affinity carrier (Chapman and Meeks 1983) or one single system (Flores and Muro-Pastor, 1988) for the uptake of glutamate and glutamine, and both amino acids may be transferred from the medium by the same permease (Lee-Kaden and Simonis, 1982; Chapman and Meeks, 1983). Clearly, cyanobacteria do not discriminate between glutamine and glutamate to such an extent as found for cyanelles in the present study. To our knowledge, the effects of 2-OG on glutamine uptake have not been studied for cyanobacteria.

The situation is also different in cyanelles and in chloroplasts of higher plants. Whereas glutamate poorly penetrates into cyanelles and does not leave them after its formation inside by the GOGAT reaction (this investigation), glutamate is believed to be the N-compound which passes the chloroplast envelope (Gimmler et al., 1974; Lehner and Heldt, 1978; Woo et al., 1987a). Glutamate is apparently transported by the dicarboxylate carrier which also transports malate and oxaloacetate in chloroplasts (Flügge et al., 1988). The present findings corroborate earlier data (Schlichting et al., 1990) that the dicarboxylate carrier does not exist in cyanelles. Glutamine is transported with low rates by the dicarboxylate carrier in chloroplasts and it is suggested that glutamine is not an important compound in the export of nitrogen from chloroplasts (Gimmler, et al., 1974; Barber and Thurman, 1978; Woo and Osmond, 1982). The glutamine carrier of cyanelles apparently has no counterpart elsewhere particularly with respect to its inhibition by glutamate.

The findings of the present investigation are in line with previous ones (Floener et al., 1982) that nitrate reductase occurs solely in the eukaryotic host whereas nitrite reductase resides in the cyanelles. The opposite claim, that both enzymes are constituents of the eukaryotic host (Böttcher et al., 1982), appears to be unlikely for several reasons: In experiments with intact cells, nitrite reduction was found to be strictly light-dependent (Bothe and Floener, 1978). Light was apparently required for the generation of reduced ferredoxin by the photosynthetic electron transport in the cyanelles. In most organisms including higher plants and free-living cyanobacteria, nitrite reduction is ferredoxin-dependent (Guerrero et al., 1981). In contrast to the situation in chloroplasts, the ferredoxin gene is encoded on the cyanelle DNA (Bayer and Schenk, 1989; Neumann-Spallart et al., 1990; Bryant et al., 1991). Thus the situation would be different in *Cyanophora* from that in other photosynthetic organisms and could not be reconciled with the location of ferredoxin if nitrite reductase were, indeed, to occur in the eukaryotic host. Ammonia formed by nitrite reduction inside the cyanelles is then incorporated by the GS reaction, and glutamine could then be exported either independently or jointly with oxoglutarate as found in the present investigation (see Fig. **5**).

The glutamine and 2-OG-supported O_2 evolution described here for cyanelles was also described for chloroplasts and attributed to ferredoxin-dependent GOGAT (Anderson and Done, 1977; Woo and Osmond, 1982; Dry and Wiskich, 1983). 2-OG is transported into chloroplasts by a specific permease which shows some overlapping specificities with the dicarboxylate carrier but is clearly distinct from it. The 2-OG translocator in chloroplasts transports malate, succinate and others in addition to 2-OG, whereas glutamate and glutamine are transferred by the dicarboxylate carrier (Woo, 1983; Woo et al., 1987a, b; Flügge et al., 1988). The 2-OG translocator is apparently involved in the photorespiratory NH_3 fixation of chloroplasts (Woo and Osmond, 1982; Proudlove et al., 1984; Wallsgrove et al., 1986; Woo et al., 1987b; Flügge et al., 1988). The 2-OG carrier in cyanelles is different as it is virtually unaffected by malate. The addition of external NH_4Cl did not stimulate O_2 evolution by cyanelles in the

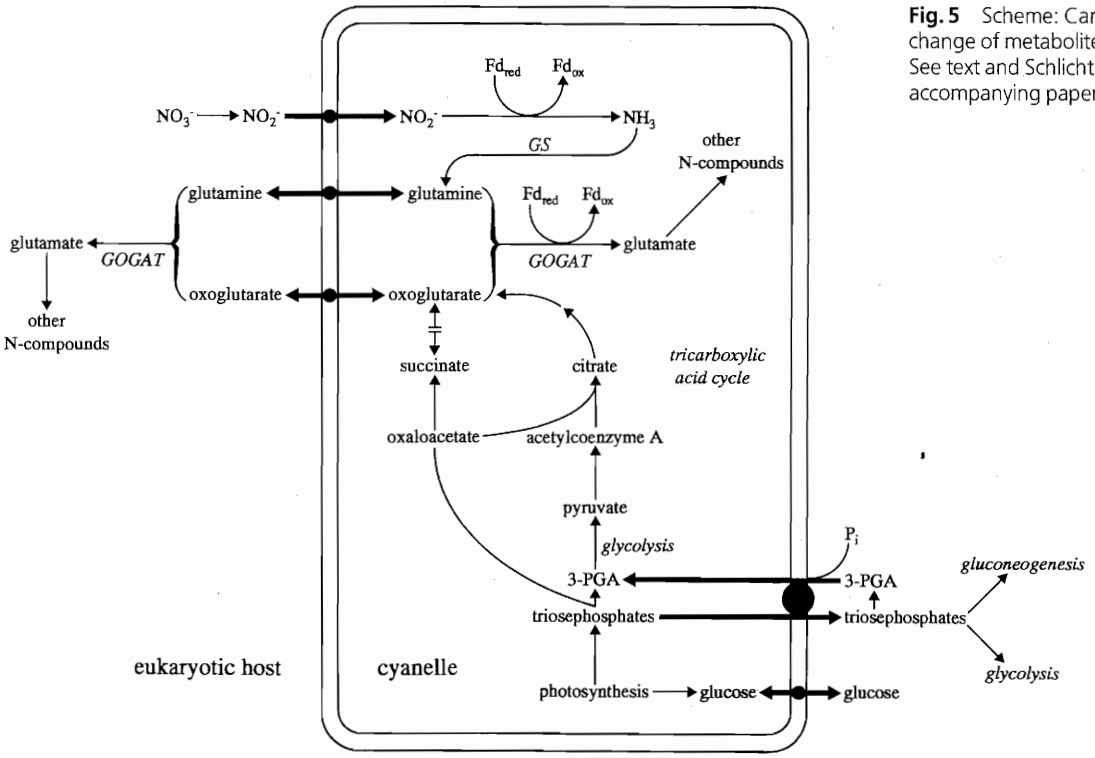

Fig. 5 Scheme: Carriers involved in the exchange of metabolites from and to cyanelles. See text and Schlichting et al., 1993, accompanying paper.

presence of glutamate, 2-OG and other compounds (Table 2). Although photorespiration was shown to occur in cyanelles (Betsche et al., 1992), the 2-OG carrier does not seem to play a specific role in this process. The 2-OG permease described here for cyanelles is also not more closely related to the 2-OG carrier of mitochondria, as this carrier also transports malate (Genchi et al., 1991).

It had been suggested that ammonia is the N-compound which leaves cyanelles (Kies and Kremer, 1990), however, this is without any experimental evidence and in analogy to the situation in *Rhizobium* and other N_2-fixing organisms. The present findings do not support this view. A transport of free ammonia could be dangerous for a photosynthetic organelle as this compound could act as uncoupler of photosynthetic electron transport. An export of glutamate is also unlikely, as cyanelles take up this amino acid only at low rates. In addition, the inhibitor experiments clearly indicated that the labeling from glutamine enriches as glutamate inside the cyanelles due to the impermeability (or low permeability) of this latter amino acid. The export of glutamine described here appears to be unusual, but is in line with the findings that this amino acid plays a specific role in heterocyst differentiation and in the transfer of nitrogen from heterocysts to vegetative cells in filamentous cyanobacteria (Wolk, 1982).

It is not yet clear whether glutamine and oxoglutarate are transported out of cyanelles by a single carrier or by separate ones. Coupling of the transport of both components in the outward direction makes sense physiologically for cyanelles (Fig. 5). Photosynthesis provides triosephosphates in these organelles. The information about carbon catabolism in cyanelles is meager, but the data so far available (Rostami-Rabet, 1980; Kaufmann, 1987) indicate that triosephosphates are degraded via glycolysis and the tricarboxylic acid cycle to 2-OG. There are some indications that *Cyanophora* cleaves pyruvate by a pyruvate dehydrogenase complex and not a pyruvate : ferredoxin oxidoreductase as filamentous cyanobacteria (Schmitz et al., 1993) and that the cyanelles are unable to degrade 2-OG (Kies and Kremer, 1990). Such an incomplete tricarboxylic acid cycle is a common feature of many obligate autotrophs, including cyanobacteria (Stanier and Cohen-Bazire, 1977). If carbon catabolism stops at 2-OG inside the cyanelles, this compound could either be exported or converted to glutamate with glutamine by the GOGAT reaction. The demand of glutamate for biosyntheses inside the cyanelles could decide whether glutamine and 2-OG are consumed inside or exported jointly (Fig. 5). Such a joint transfer requires fine regulation between photosynthesis/carbon catabolism and assimilatory nitrate reduction/glutamate synthesis. The regulatory components involved are unknown. Thioredoxin is apparently not involved as this protein was recently shown not to occur in cyanelles (Dai et al., 1992).

The transport of an acid dicarboxylic acid together with a basic amino acid could be electroneutral, depending on the ionic properties of the translocator(s) involved. The overall activity of light and 2-OG-stimulated uptake of glutamine into cyanelles amounts 5–8 nmol min^{-1} mg chl^{-1} (determined from the ^{14}C-glutamine uptake experiments) and is thus considerably lower than the transport rates observed with isolated plant chloroplasts. It should, however, be kept in mind that the generation time of *Cyanophora paradoxa* is 20–24 h. In addition, the CO_2 fixation rate of isolated cyanelles is about 40 nmol CO_2 fixed min^{-1} mg chl^{-1} within the first few min (a value which was also observed independently of this laboratory by Kleinig et

al., 1986) and the activity of the phosphate translocator is also about 30 nmol P_i transferred min^{-1} mg chl^{-1} (Schlichting and Bothe, 1993). Thus the activities of cyanelles are in general much lower than in chloroplasts. To our knowledge, a coupling of an export of glutamine and 2-OG has not been described for any organelle or organism. In conclusion, cyanelles appear to have unusual features particularly with respect to their glutamine and glutamate transport.

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