Biochem. J. (1992) 282, 639–643 (Printed in Great Britain)

Nitrate transport in the cyanobacterium *Anacystis nidulans* R2

Kinetic and energetic aspects

Rocío RODRÍGUEZ, Catalina LARA* and Miguel G. GUERRERO
Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Apdo. 1113, 41080-Sevilla, Spain

Nitrate transport has been studied in the cyanobacterium *Anacystis nidulans* R2 by monitoring intracellular nitrate accumulation in intact cells of the mutant strain FM6, which lacks nitrate reductase activity and is therefore unable to reduce the transported nitrate. Kinetic analysis of nitrate transport as a function of external nitrate concentration revealed apparent substrate inhibition, with a peak velocity at 20–25 μM-nitrate. A K_s (NO_3^-) of 1 μM was calculated. Nitrate transport exhibited a stringent requirement for Na^+. Neither Li^+ nor K^+ could substitute for Na^+. Monensin depressed nitrate transport in a concentration-dependent manner, inhibition being more than 60% at 2 μM, indicating that the Na^+-dependence of active nitrate transport relies on the maintenance of a Na^+ electrochemical gradient. The operation of an Na^+/NO_3^- symport system is suggested. Nitrite behaved as an effective competitive inhibitor of nitrate transport, with a K_i (NO_2^-) of 3 μM. The time course of nitrite inhibition of nitrate transport was consistent with competitive inhibition by mixed alternative substrates. Nitrate and nitrite might be transported by the same carrier.

INTRODUCTION

Nitrate transport into the cell is the first and least understood step in the nitrate-assimilation process. Operativity of nitrate transport determines substrate availability for nitrate reductase and represents the first potential control point of nitrate assimilation (Guerrero *et al.*, 1990).

Entrance of nitrate in cyanobacterial cells has been conventionally studied by monitoring uptake of the ion from the external medium, and the process has been shown to exhibit saturation kinetics with K_s values for nitrate below 50 μM (Zevenboom *et al.*, 1980; Flores *et al.*, 1983; Tischner & Schmidt, 1984). Such an approach does not, however, allow one to discriminate transport itself from the subsequent metabolism of nitrate. A sensitive method has recently been developed to measure intracellular nitrate levels and applied to the study of nitrate transport in the unicellular cyanobacterium *Anacystis nidulans* (Lara *et al.*, 1987; Romero *et al.*, 1989). As the intracellular nitrate level is determined by the balance between the rate of net entrance to the cell and the rate of its reduction by nitrate reductase, changes in internal nitrate concentration represent a reliable estimate of nitrate transport activity only when nitrate reductase activity is negligible. This has been accomplished by tungstate treatment of *Anacystis* to generate cells with reduced levels of nitrate reductase (Herrero & Guerrero 1986; Lara *et al.*, 1987) or by assaying in the dark (Sivak *et al.*, 1989; Guerrero *et al.*, 1990), where the lack of photogenerated reductant leads to negligible nitrate reduction *in vivo*. These approaches have allowed one to demonstrate the operativity of an active nitrate transport system in *A. nidulans* subject to metabolic control by products of nitrogen assimilation (Lara *et al.*, 1987) and to establish a close parallelism between the nitrate-transport capacity of the cells and the amount of a 47 kDa plasma-membrane polypeptide that is presumed to be involved in nitrate transport (Sivak *et al.*, 1989; Guerrero *et al.*, 1990).

In the search for an appropriate system in which nitrate-transport activity can be characterized without the drawbacks and side effects of tungstate treatment of the cells or the limitation in energy supply of darkened cells (Guerrero *et al.*, 1990), a mutant strain of *A. nidulans* R2 named 'FM6', which lacks nitrate reductase activity (Madueño *et al.*, 1988), has been selected for kinetic and bioenergetic analysis of nitrate transport. The results of this study are reported here.

MATERIALS AND METHODS

Organism and culture conditions

Anacystis nidulans R2 (*Synechococcus elongatus* PCC 7942) was grown photoautotrophically at 40 °C with nitrate as nitrogen source as described by Madueño *et al.* (1988). The mutant strain FM6, which lacks nitrate reductase activity, was grown under the same conditions, but with 7.5 mM-$(NH_4)_2SO_4$ as the nitrogen source, to a cell density equivalent to 20 μg of chlorophyll *a*/ml. For induction of nitrate transport (Sivak *et al.*, 1989), an amount of cells equivalent to 150 μg of chlorophyll *a* was harvested by centrifugation (8000 *g*, 10 min, room temperature) and transferred to a medium containing 4 mM-KNO_2 as the nitrogen source. After 18 h, the cells were harvested by centrifugation, washed and resuspended in the buffer used for the nitrate-transport assays. Chlorophyll *a* was determined after methanol extraction as described by McKinney (1941).

Nitrate-transport assay

Nitrate-transport activity was determined by measuring intracellular nitrate accumulation in acid lysates of cells subject to silicone-oil centrifugation as described by Romero *et al.* (1989), with the following modifications. The nitrate-transport assay was carried out at 40 °C in air-opened conical flasks with continuous shaking and illumination. For standard assays, the medium (5 ml) contained 25 mM-Tricine buffer, adjusted at pH 8.3 at 40 °C with an equimolar solution of NaOH and KOH, 10 mM-$NaHCO_3$, an amount of cells equivalent to 33 μg of chlorophyll *a*/ml and a variable concentration of $NaNO_3$. For assays of cation requirement the medium contained 25 mM-Tricine/90 mM-Tris buffer, pH 8.3 (at 40 °C), 20 μM-HNO_3, 500 μM of the chloride salt of the corresponding univalent cation and an amount of cells equivalent to 33 μg of chlorophyll *a*/ml. The assay was started by simultaneous addition of nitrate and

* To whom correspondence should be addressed.

illumination. At the required times, aliquots (0.3 ml) were rapidly transferred to 0.4 ml polyethylene microcentrifuge tubes containing 0.02 ml of 20 mM-sulphonic acid in 2 M-H_3PO_4 and the silicone layer [0.08 ml of a 2:1 (v/v) mixture of Versilube F50 (Serva) and silicone 14615-3 (Aldrich)]. After rapid centrifugation (10000 *g*, 1 min) in a Beckman Microfuge 11, nitrate was analysed in aliquots of the acid cell lysate by ion-exchange h.p.l.c. as previously described (Romero *et al.*, 1989). Intracellular nitrate concentrations have been calculated for an aqueous internal volume for *A. nidulans* cells of 100 μl/mg of chlorophyll *a* (Miller & Canvin, 1985).

Monensin treatment was performed by preincubating the cells with the inhibitor for 5 min in the dark before the assay. Monensin was dissolved in ethanol. Adequate controls containing the same ethanol concentration were run.

Kinetics of nitrate transport

At the low concentrations of external nitrate used in the transport assays, a substantial fraction of the substrate is utilized during the assay, from 7% at 20 μM-nitrate to 50% at 2 μM-nitrate after 2 min. The provision was therefore made to relate the initial rate of nitrate transport, taken as the mean velocity ($\bar{v}$) during the initial 2 min period, to the corresponding mean substrate concentration ($[\bar{S}] = \frac{1}{2}([S]_0 + [S])$ over this period (Segel, 1975).

RESULTS

Nitrate accumulation by intact cells of *Anacystis nidulans* R2 FM6

When exposed to 20 μM external nitrate, illuminated cells of the wild-type strain *Anacystis nidulans* R2 did not accumulate nitrate intracellularly at measurable levels, reflecting a steady-state situation in which nitrate transport and reduction take place simultaneously. Under the same conditions, however, cells of the mutant strain FM6, which lacks nitrate reductase activity (Madueño *et al.*, 1988), exhibited intracellular nitrate levels above 600 μM. A typical time course of nitrate accumulation by illuminated cells of the FM6 mutant is shown in Fig. 1. Intracellular nitrate increased linearly with time to reach in 3–4 min a plateau that remained steady or, in some cases (see below), decreased after 5–6 min. Considering that the plateau concentration corresponds to an equilibrium situation, and assuming a membrane potential of −100 mV (Paschinger, 1977), the ΔG for the achievement and maintenance of such intracellular nitrate concentrations, calculated according to the Nernst equation, amounts to about +20 kJ/mol of nitrate. This illustrates the strongly endergonic nature of nitrate transport in cyanobacterial cells.

In darkness, when nitrate reduction in the wild-type strain was also limited by provision of reductant, cells of both wild-type and FM6 mutant accumulated nitrate to comparable levels, about 200–300 μM, when exposed to 20 μM external nitrate (results not shown). The similar nitrate accumulation pattern exhibited by both strains in darkness indicates an analogous nitrate transport capacity for the FM6 mutant and the wild-type strain, which supports the selection of the FM6 strain as an appropriate and useful system for the kinetic characterization of nitrate transport.

Kinetics of nitrate transport

Initial mean velocities of net nitrate transport as a function of external nitrate concentration are plotted in Fig. 2. The nitrate-transport rate increased in a hyperbolic fashion in response to increasing external nitrate, to reach a maximum at 20–25 μM, and decreased for higher nitrate concentrations. This kinetic pattern is indicative of substrate inhibition, as also evidenced the double-reciprocal plot of the data (inset to Fig. 2), with a deviation upwards of the data points as they approached the vertical axis. From the linear part of the plot, an apparent K_s (NO_3^-) of 1 μM was calculated. The intracellular nitrate concentration had no apparent effect on the initial velocity of net nitrate transport, indicating that product inhibition might be negligible.

Nitrate-transport activity in the FM6 mutant cells exhibited an optimum temperature of 40 °C (results not shown), which was the temperature used for growth of the cells. A Q_{10} (30–40) value of 3, very similar to that reported for nitrate uptake in *Chlorella sorokiniana* (Tischner & Lorenzen, 1981) and nitrate transport in barley (*Hordeum vulgare*) roots (Glass *et al.*, 1990) was calculated. This Q_{10} value corresponds to an activation energy of 87 kJ/mol of nitrate.

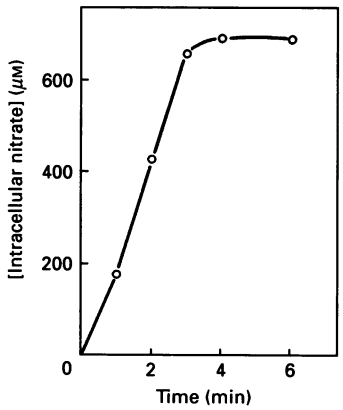

Fig. 1. Time course of intracellular nitrate accumulation in intact cells of *Anacystis nidulans* R2 FM6 mutant

Nitrate transport was assayed under illumination in the presence of 20 μM-$NaNO_3$ at saturating Na^+ concentrations.

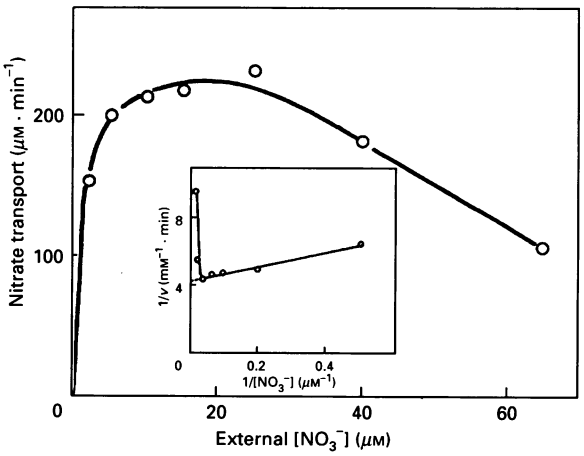

Fig. 2. Effect of external nitrate concentration on the initial velocity of nitrate transport

Initial mean velocities of nitrate transport, assayed at saturating Na^+ concentrations, were plotted against mean substrate concentrations. The inset represents a double-reciprocal plot of the data.

Na^+-dependence of nitrate transport

Nitrate transport exhibited a strong and selective requirement for Na^+. As Fig. 3 shows, very low intracellular nitrate was accumulated in the absence of univalent cations. Addition of Na^+ markedly stimulated nitrate-transport activity, whereas either Li^+ or K^+ were without effect, intracellular nitrate accumulation being as low as that measured in the absence of added cations. It is noteworthy that, at suboptimal concentrations of Na^+, such as that used in the experiment shown in Fig. 3 (500 μM), a significant decrease in intracellular nitrate was observed after 3–4 min. Maintenance of maximal intracellular nitrate levels (as in Fig. 1) required millimolar Na^+ concentrations. In the absence of added Na^+, dissipation of intracellular nitrate accumulation took place very rapidly, indicating nitrate efflux following the nitrate electrochemical gradient. Furthermore, nitrate-transport activity was sensitive to monensin, an ionophore which relaxes the sodium electrochemical gradient (Skulachev, 1985; Brown *et al.*, 1990). Preincubation of cells with increasing monensin concentrations led to depression of nitrate-transport activity, the inhibition being higher than 60% for monensin concentrations as low as 2 μM (Fig. 4). The monensin effect indicates that nitrate-transport activity relies on the maintenance of a sodium electrochemical gradient across the plasmalemma, which might represent the immediate source of energy for active nitrate transport.

Nitrite inhibition of nitrate transport

Nitrite, an alternative nitrogen source, behaved as an effective competitive inhibitor of nitrate transport. Fig. 5 shows that double-reciprocal plots of initial mean velocities versus sub-inhibitory nitrate concentrations, in the absence and presence of nitrite, intercepted the $1/v$ axis on the same point. A K_i value for nitrite of 3 μM was estimated. This indicates that nitrite binds to the nitrate transporter with very high affinity, analogous to that shown by nitrate (K_s 1 μM). Nitrite did not affect the substrate-inhibition pattern of nitrate transport.

When nitrate-transport activity was assayed at a constant nitrate concentration, inhibition of nitrate transport by added nitrite at concentrations up to 40 μM was more marked at the

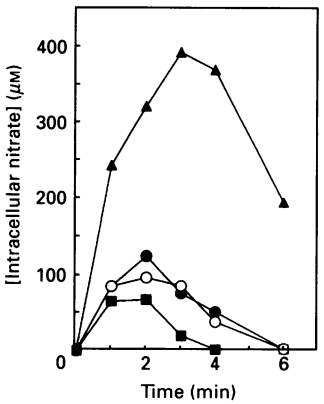

Fig. 3. Effect of univalent cations on nitrate transport in *A. nidulans* R2 FM6 strain

Nitrate transport was assayed at 20 μM-HNO_3 in Tris/Tricine buffer containing: no added salts (○), LiCl at 500 μM (●), NaCl at 500 μM (▲) or KCl at 500 μM (■).

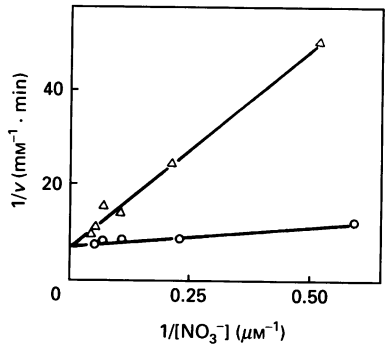

Fig. 5. Effect of nitrite on nitrate-transport rate

A double-reciprocal plot of initial mean velocities versus sub-inhibitory nitrate concentrations, in the absence (○) or presence (△) of 30 μM-KNO_2, is shown.

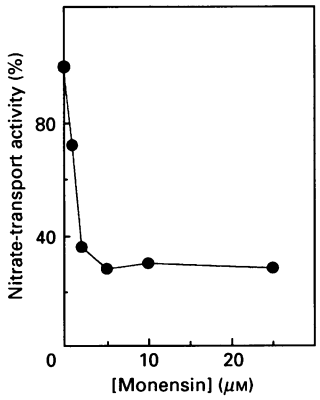

Fig. 4. Concentration-dependent effect of monensin on nitrate-transport activity in *A. nidulans* R2 FM6 strain

Nitrate transport was assayed at saturating Na^+ concentrations and 20 μM-nitrate. 100% of activity in the control corresponded to an intracellular nitrate accumulation of 1480 μM after 5 min of assay.

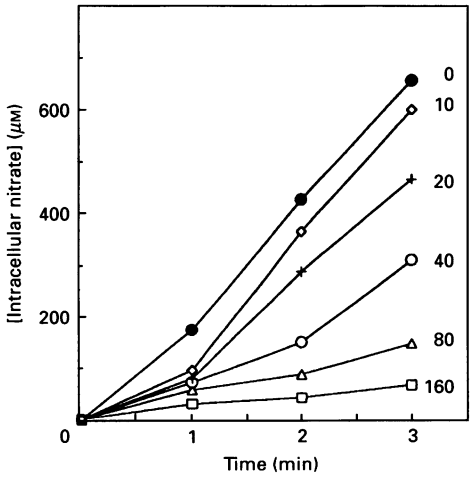

Fig. 6. Effect of nitrite concentrations on the time course of intracellular nitrate accumulation

Nitrate-transport activity at saturating Na^+ concentrations was determined at 20 μM-$NaNO_3$ in the absence or presence of KNO_2 at the indicated (μM) concentrations.

beginning of the assay and decreased progressively thereafter (Fig. 6). At higher nitrite concentrations the inhibition of nitrate transport was maintained throughout the assay. Such a behaviour is exhibited by systems which are competitively inhibited by mixed alternative substrates (Segel, 1975). As such, nitrite would be transported and used by the cells, its external concentration being rapidly decreased to reach levels no longer inhibitory for nitrate transport. The data thus indicate that nitrate and nitrite might be transported by the same carrier in *Anacystis*.

DISCUSSION

The strain used in this study, the FM6 mutant of *Anacystis nidulans* R2, which lacks nitrate reductase activity (Madueño *et al.*, 1988), has a nitrate-transport activity comparable with that of the wild-type strain, and to that of the closely related species *A. nidulans* (*Synechococcus leopoliensis* 1402–1) (Lara *et al.*, 1987; Sivak *et al.*, 1989; Guerrero *et al.*, 1990). As such, nitrate transport in the FM6 strain is strongly endergonic, with ΔG values similar to those previously reported for tungstate-treated *A. nidulans* cells (Lara *et al.*, 1987). Moreover, nitrate accumulation by cells of FM6 is also subject to nutritional repression by NH_4^+ as well as to rapid inhibition by ammonium assimilation products (R. Rodriguez, C. Lara & M. G. Guerrero, unpublished work). These similarities allow one to derive essential information about the characteristics of nitrate transport in *Anacystis* from studies performed in this mutant strain.

At saturating Na^+ concentrations, nitrate transport exhibited hyperbolic kinetics, with a peak velocity corresponding to apparent substrate inhibition. Other authors have reported substrate inhibition of nitrate uptake, determined by monitoring nitrate disappearance from the external medium, in *A. nidulans* (Tischner & Schmidt, 1984) and *Chlorella sorokiniama* (Tischner & Lorenzen, 1981) and have suggested that binding of two nitrate ions to the nitrate transporter would result in an inactive complex. The Na^+ requirement for nitrate transport reported here provides the basis for interpreting the apparent substrate inhibition of nitrate transport in terms of a symport of nitrate and Na^+. Bisubstrate enzymes and carriers can exhibit apparent substrate inhibition depending on the reaction mechanism (Segel, 1975; Cleland, 1979), this phenomenon being specially frequent for systems involving metal ions (London & Steck, 1969; Morrison, 1979). Several reaction mechanisms could account for the apparent substrate inhibition by nitrate, including that of $NaNO_3$ being the true substrate for the carrier (London & Steck, 1969). Although more detailed kinetic studies are needed to unravel the role of Na^+ in nitrate transport, the monensin effect (Fig. 4) also indicates the energy dependence of nitrate transport on the Na^+ electrochemical gradient across the plasma membrane. In this regard, the possibility of nitrate and Na^+ as co-substrates for the nitrate transporter would provide a physical basis for the energetic coupling between endergonic nitrate transport and the Na^+ electrochemical gradient.

Interestingly it has been reported that illuminated cyanobacteria actively excrete Na^+ through an Na^+/H^+ antiporter (Blumwald *et al.*, 1984; Molitor *et al.*, 1986) or a primary light-dependent Na^+ pump (Brown *et al.*, 1990). As a result, the cells maintain very low intracellular Na^+ concentrations (Dewar & Barber, 1973) and a downhill Na^+ electrochemical gradient from outside to inside the cells, which is abolished by monensin (Brown *et al.*, 1990). The light-dependence of Na^+ extrusion in cyanobacteria could explain the lower nitrate transport activities observed in the dark in the FM6 mutant as well as in *A. nidulans* cells (Guerrero *et al.*, 1990). Active bicarbonate transport in cyanobacteria has also been shown to be dependent on millimolar concentrations of Na^+ (Kaplan *et al.*, 1984; Espie *et al.*, 1988; Miller *et al.*, 1990). The Na^+-dependence for the transport of both bicarbonate and nitrate provides the basis for the well-known essentiality of Na^+ for cyanobacterial growth at alkaline pH (Kratz & Myers, 1955; Miller *et al.*, 1984) and reinforce the importance of Na^+ energetics in cyanobacteria (Skulachev, 1985).

The kinetics of nitrite inhibition of nitrate transport in the FM6 mutant strongly support the contention that nitrate and nitrite are transported by the same transport system. This is in agreement with the reported competitive inhibition by nitrate of nitrite uptake in *A. nidulans* (Madueño *et al.*, 1987) and in the eukaryotic microalgae *Phaeodactylum tricornutum* (Bilbao *et al.*, 1981) and *Cyanidium caldarium* (Fuggi, 1989). In other microalgae the existence of two different transport systems for nitrate and nitrite has been proposed, however, on the basis of mixed competitive inhibition by nitrite of nitrate uptake (Córdoba *et al.*, 1986) and differential pH-dependence of nitrate and nitrite uptakes (Ullrich, 1983). The results presented here clearly show the competitive nature of nitrite inhibition of nitrate transport and the similar affinity of the carrier for both nitrate (K_s, 1 μM) and nitrite (K_i, 3 μM), consistent with its operativity as a nitrate/nitrite transporter in *Anacystis* cells.

This research has been financed by grants from Dirección General de Inestigación Científica y Técnica (PB88-0019) and Plan Andaluz de Investigación (Spain). We thank Dr. J. M. Romero for his collaboration in the early stages of this work and Dr. F. Madueño for kindly making available the FM6 strain. The skilful secretarial assistance of P. Pérez de León is gratefully acknowledged.

REFERENCES

Bilbao, M. M., Gabas, J. M. & Serra, J. L. (1981) Biochem. Soc. Trans. **9**, 476–477
Blumwald, E., Wolosyn, J. M. & Packer, L. (1984) Biochem. Biophys. Res. Commun. **122**, 452–459
Brown, I. I., Fadeyev, S. I., Kirik, I. I., Severina, I. I. & Skulachev, V. P. (1990) FEBS Lett. **270**, 203–206
Cleland, W. W. (1979) Methods Enzymol. **63**, 500–513
Córdoba, F., Cárdenas, J. & Fernández, E. (1986) Plant Physiol. **82**, 904–908
Dewar, M. A. & Barber, J. (1973) Planta **113**, 143–155
Espie, G. S., Miller, A. G. & Canvin, D. T. (1988) Plant Physiol. **88**, 757–763
Flores, E., Guerrero, M. G. & Losada, M. (1983) Biochim. Biophys. Acta **722**, 408–416
Fuggi, A. (1989) Plant Physiol. Biochem. **27**, 563–568
Glass, A. D. M., Siddiqi, M. Y., Ruth, T. J. & Rufty, T. W., Jr. (1990) Plant Physiol. **93**, 1585–1589
Guerrero, M. G., Romero, J. M., Rodríguez, R. & Lara, C. (1990) in Inorganic Nitrogen in Plants and Microorganisms (Ullrich, W. R., Rigano, C., Fuggi, A. & Aparicio, P. J., eds.), pp. 79–85, Springer, Berlin
Herrero, A. & Guerrero, M. G. (1986) J. Gen. Microbiol. **132**, 2463–2486
Kaplan, A., Volokita, M., Zenvirth, D. & Reinhold, L. (1984) FEBS Lett. **176**, 166–168
Kratz, W. A. & Myers, J. (1955) Am. J. Bot. **42**, 282–287
Lara, C., Romero, J. M. & Guerrero, M. G. (1987) J. Bacteriol. **169**, 4376–4378
London, W. P. & Steck, T. L. (1969) Biochemistry **8**, 1767–1779
McKinney, G. (1941) J. Biol. Chem. **140**, 315–322
Madueño, F., Flores, E. & Guerrero, M. G. (1987) Biochim. Biophys. Acta **896**, 109–112
Madueño, F., Borrias, W. E., Van Arkel, G. A. & Guerrero, M. G. (1988) Mol. Gen. Genet. **213**, 223–228
Miller, A. G. & Canvin, D. T. (1985) FEBS Lett. **187**, 29–32
Miller, A. G., Turpin, D. H. & Canvin, D. T. (1984) J. Bacteriol. **159**, 100–106
Miller, A. G., Espie, G. S. & Canvin, D. T. (1990) Can. J. Bot. **68**, 1291–1302
Molitor, V., Erber, W. & Peschek, G. A. (1986) FEBS Lett. **204**, 251–256
Morrison, J. F. (1979) Methods Enzymol. **63**, 257–294
Paschinger, H. (1977) Arch. Microbiol. **113**, 285–291

Romero, J. M., Lara, C. & Guerrero, M. G. (1989) Biochem. J. **259**, 545–548
Segel, I. H. (1975) Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, Wiley-Interscience, New York
Sivak, M. N., Lara, C., Romero, J. M., Rodríguez, R. & Guerrero, M. G. (1989) Biochem. Biophys. Res. Commun. **158**, 257–262
Skulachev, V. P. (1985) Eur. J. Biochem. **151**, 199–208
Tischner, R. & Lorenzen, H. (1981) Biology of Inorganic Nitrogen and Sulfur (Bothe, H. & Trebst, A., eds.), pp. 252–259, Springer, Berlin
Tischner, R. & Schmidt, A. (1984) Arch. Microbiol. **137**, 151–154
Ullrich, W. R. (1983) Encycl. Plant Physiol. New Ser. **15A**, 376–397
Zevenboom, W., De Groot, G. J. & Mur, L. R. (1980) Arch. Microbiol. **125**, 59–65

Received 1 August 1991/26 September 1991; accepted 3 October 1991