Separating the efflux and influx components of net nitrate uptake by *Synechococcus* R2 under steady-state conditions

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N isotopic discrimination was used to determine the ratio of influx to efflux in experiments in which NO_3^- reduction was decreased (and also net NO_3^- uptake, since under steady-state conditions, net NO_3^- uptake is equal to the rate of reduction) by tungstate. Exponential growth rate constants for the cyanobacterium *Synechococcus* sp. R2 grown in media containing $0.18 \mu\text{M}$ molybdate decreased linearly (0.040 to 0.013 h^{-1}) with increasing tungstate concentration (0 to 0.2 mM). Values of the overall, observed N isotope effect (β_{obs} , the ratio of the rate of reaction of ^{14}N - to ^{15}N -bearing molecules, normalized to adjust for unequal concentrations) varied inversely and were linear with exponential growth rate. At high tungstate concentration, when the exponential growth rate approached zero (as a result of negligible NO_3^- reduction), β_{obs} approached 1.0197 . In the absence of tungstate, β_{obs} was equal to 1.0037 . From this value, we calculated the ratio of efflux to influx to be about 0.19 . That is, in the absence of tungstate, more than 80% of the NO_3^- entering the cell underwent reduction with less than 20% released to the medium from the cell. This approach can be used to distinguish changes in influx from changes in metabolic steps in response to factors which affect net uptake rate.

Introduction

Considerable information is available on factors affecting assimilation of NO_3^- by *Synechococcus* sp. R2 (PCC 7942). In this organism, reduction of NO_3^- and NO_2^- , as well as CO_2 , requires the photochemically generated electron donor reduced ferredoxin (Candau *et al.*, 1976; Lara & Romero, 1986). For this reason, and also because active CO_2 fixation is independently required, presumably to provide carbon skeletons for N assimilation (Romero *et al.*, 1985), NO_3^- assimilation is dependent on light intensity (Lara & Romero, 1986). Utilization of NO_3^- is inhibited by NH_4^+ (Flores *et al.*, 1980; Herrero *et al.*, 1981) as well as by DL-glyceraldehyde (DLG), an inhibitor of CO_2 fixation (Romero *et al.*, 1985). Short-term inhibition by NH_4^+ of NO_3^- utilization occurs without effect on nitrate reductase (NR) activity (Flores *et al.*, 1980). However, in the long-term, NR activity is inhibited (Herrero *et al.*, 1981). Restoration of activity is inhibited by rifampicin, indicating transcriptional control of NR synthesis by NH_4^+ .

Negative effectors may act either by decreasing NO_3^- influx or by decreasing the rate of metabolic steps. In short-term experiments Lara *et al.* (1987) provided the

following evidence that NH_4^+ and DLG act on the uptake step. Molybdenum is required for the synthesis of active NR (Candau, 1979). Because tungsten is a structural analogue of molybdenum, substitution of tungstate for molybdate in the growth medium results in the synthesis of inactive NR. Lara *et al.* (1987) demonstrated active transport of NO_3^- in cells with tungstate-inactivated NR. Intracellular NO_3^- concentrations in such cells were four to eight times higher than in the medium 60 s after exposure to NO_3^- . No accumulation occurred in the presence of NH_4^+ or DLG, indicating that these inhibitors act on the uptake system. However, long-term exposure to NH_4^+ must have an effect beyond the uptake step, since active NR (the synthesis of which does not require the presence of NO_3^-) is not synthesized under these conditions (Herrero *et al.*, 1981).

It would be of considerable interest to determine the relative degree to which negative (or positive) effectors of NO_3^- utilization exert their effect through changes in influx or metabolism. Present methods do not permit such a determination under steady-state conditions. Measurements of influx, as opposed to net uptake rate, usually involve short-term experiments in which the rate of transport of labelled substrate (or labelled substrate analogue) from the medium into cells is determined. Such measurements provide transient values for influx, which may be different from influx after a steady-state

Abbreviations: β_{obs} , overall, observed N isotope effect; DLG, DL-glyceraldehyde; NR, nitrate reductase.

has been established. Under steady-state conditions, NO_3^- influx is equal to the rate of NO_3^- metabolism plus efflux, with the net uptake rate being equal to the rate of NO_3^- metabolism (i.e., the rate of metabolism is equal to influx minus efflux). Therefore, if it were possible to measure the ratio of efflux to influx, this ratio, together with a measured value for net uptake rate would permit calculation of the absolute values of influx and efflux. In this paper we report the positive result of a feasibility test of this approach to separating the influx and efflux components of net NO_3^- uptake.

Methods

Synechococcus sp. R2 was obtained from L. Sherman (Purdue University, West Lafayette, IN, USA). Cultures were maintained on 1.5% (w/v) agar BG-11 medium (Rippka *et al.*, 1979), with 5 mM- NaNO_3 . Experimental cultures were grown in 800 ml or 100 ml of liquid medium BG-11 containing 2 mM- or 5 mM- NaNO_3 . Liquid cultures were kept in an illuminated (two 40 W daylight fluorescent strips at a distance of 21 cm) water-bath at 30 °C, and bubbled with a mixture of 5% (v/v) CO_2 in air. Growth was followed by measuring the optical density at 750 nm.

After growth for an appropriate time, samples were collected and cells removed by centrifugation for 10 min at 10000 g. In order to determine the relationship between OD_{750} and protein concentration, cells at various growth stages were washed twice with 100 mM- NaHCO_3 and 0.8 mM- MgSO_4 , adjusted to pH 7.4. Washed cells were subjected to Kjeldahl digestion followed by distillation of the NH_4^+ in the digest into boric acid containing a mixed indicator, and titration with standard HCl (Shearer & Kohl, 1986). Protein was assumed to contain 16% (w/w) N (Bergersen, 1980). The correlation coefficient between OD_{750} and protein concentration was 0.98, with the slope corresponding to 200 μg protein (OD unit^{-1}). Loss of NO_3^- from the medium was measured by steam distillation techniques (Bremner & Keeney, 1965). Any NH_4^+ which might have been present was separated from the sample by steam distillation (in the presence of MgO) prior to reducing NO_3^- . No free NH_4^+ was detected in any of the samples. We considered it unnecessary to remove NO_2^- prior to reducing NO_3^- . Although NO_2^- has been shown to accumulate when *Synechococcus* 6301 is incubated under conditions of C or S deficiency (Krämer & Schmidt, 1989), it did not accumulate under the conditions we used (5% CO_2 in the gas stream and 300 μM - MgSO_4 in the medium). After reducing NO_3^- to NH_4^+ with Devarda's alloy, the resulting NH_4^+ was distilled. The amount of N in the distillate was determined by titration. After acidification and evaporation of the distillate, the N derived from NO_3^- was converted to N_2 in an evacuated Sprinson-Rittenberg tube by reaction with NaOBr (Shearer & Kohl, 1986). Isotope analysis was performed on the resulting N_2 using an isotope ratio mass spectrometer equipped with dual collectors and a dual inlet system (VG Micromass 602E). The amount of NO_3^- -N lost from the medium was equal to the amount of N accumulation in the cells, indicating complete recovery of added N.

Results and Discussion

The principle of using isotopic discrimination for measuring efflux/influx

The overall, observed N isotope effect (isotopic fractionation factor), β_{obs} , is defined as the rate of reaction of

^{15}N -bearing molecules, relative to the rate of reaction of ^{14}N -bearing molecules, where both rates are normalized to adjust for unequal concentrations of the two kinds of molecules.

$$\beta_{\text{obs}} = \frac{d[^{14}\text{N}]/dt}{[^{14}\text{N}]} \bigg/ \frac{d[^{15}\text{N}]/dt}{[^{15}\text{N}]} \quad (1)$$

In closed systems (systems in which substrate is consumed during the course of the reaction) experimentally determined values of β_{obs} are calculated from measurements, in competitive experiments (in competitive experiments for measuring isotope effects, both the heavy and light isotopes are present in the same vessel), of the change in the natural abundance of ^{15}N of substrate at two times (time zero, and some later time), and the extent of reaction. Natural ^{15}N abundance is expressed as $\delta^{15}\text{N}$, the per mil ^{15}N excess compared to a standard (customarily atmospheric N_2).

$$\delta^{15}\text{N} = \frac{\frac{^{15}\text{N}}{^{14}\text{N}} \text{ sample} - \frac{^{15}\text{N}}{^{14}\text{N}} \text{ standard}}{\frac{^{15}\text{N}}{^{14}\text{N}} \text{ standard}} \cdot 1000\text{‰} \quad (2)$$

The overall isotopic fractionation factor is calculated from experimental measurements using the approximate expression below (which gives the same result within experimental error as the exact expression) (Mariotti *et al.*, 1981).

$$\beta_{\text{obs}} = \frac{\delta^{15}\text{N}_{s_0} - \delta^{15}\text{N}_{s_t}}{1000 \ln f} + 1 \quad (3)$$

where f is the ratio of substrate concentration at time t to substrate concentration at time zero, and the subscripts 's₀' and 's_t' refer to substrate at times zero and t , respectively.

The magnitude of β_{obs} depends, not only upon the intrinsic isotope effects (the isotope effect associated with a single, unidirectional step within a reaction sequence is referred to as an intrinsic isotope effect) associated with individual steps within the overall reaction sequence, but also upon the relative rates of the forward and reverse steps. This latter dependence can be illustrated by an expression for β_{obs} derived from the scheme shown in Fig. 1. In this scheme, k_1 , k_2 , k_3 , k_4 and k_5 are rate constants for influx, efflux, enzyme-substrate binding, enzyme-substrate dissociation and NO_3^- reduction, respectively. Some of these 'rate constants' subsume other reactants needed for the step to proceed. For example, the ATP concentration is subsumed in k_1 and k_2 if it is required for transport; the concentration of reduced ferredoxin may be subsumed in k_5 , depending on whether NR is reduced before or after NO_3^- -NR association.

Fig. 1. Simplified diagram of NO₃⁻ assimilation. NO₃⁻_(out) is NO₃⁻ in the medium surrounding the cell; NO₃⁻_(in) is NO₃⁻ inside the cell; NR represents nitrate reductase; NR·NO₃⁻ and NR·NO₂⁻ represent enzyme-bound species. Rate constants for influx, efflux, enzyme-substrate association, enzyme-substrate dissociation, and reduction are indicated by k_1 , k_2 , k_3 , k_4 , and k_5 , respectively.

The derivation of an expression for β_{obs} based on the model shown in Fig. 1 starts with the derivation of an expression for overall velocity (v). Such a derivation for an analogous five-step model has been published previously (Shearer & Kohl, 1988), and is equivalent to the expression

$$v = \frac{k_1[\text{NO}_3^-(\text{out})]PQ}{1 - P + PQ} \quad (4)$$

where P and Q represent the two partition factors of the reaction sequence shown in Fig. 1.

$$P = \frac{k_3[\text{NR}]}{k_2 + k_3[\text{NR}]} \text{ and } Q = \frac{k_5}{k_4 + k_5}$$

Thus, P represents the relative amount of NO₃⁻_(in) reacting in the forward direction, and Q represents the relative amount of enzyme-bound NO₃⁻_(in) reacting in the forward direction.

An expression for β_{obs} is derived by substituting expressions for v or ^{15}v into the definition of β_{obs} (eqn 1). The result (Shearer & Kohl, 1988) is

$$\begin{aligned} \beta_{\text{obs}} &= \frac{\frac{\beta_1\beta_3\beta_5}{\beta_2\beta_4}(1 - P)(1 - Q) + \frac{\beta_1\beta_3}{\beta_2}Q(1 - P) + \beta_1QP}{(1 - P)(1 - Q) + Q(1 - P) + QP} \\ &= \frac{\frac{\beta_1\beta_3\beta_5}{\beta_2\beta_4}(1 - P)(1 - Q) + \frac{\beta_1\beta_3}{\beta_2}Q(1 - P) + \beta_1QP}{1 - P + QP} \end{aligned} \quad (5)$$

where β_1 , β_2 , β_3 , β_4 and β_5 are intrinsic isotope effects for the indicated steps. Thus, the overall isotope effect for the reaction sequence of Fig. 1 is the weighted average of the ratios $\beta_1\beta_3\beta_5/\beta_2\beta_4$, $\beta_1\beta_3/\beta_2$ and β_1 with the weighting being related to the relative success of forward and reverse rates of the relevant steps (i.e. the partition factors P and Q).

A simplifying assumption seems justified at this point. Protein-substrate complexes are almost always held

together by electrostatic and hydrogen bonds, so that appreciable isotope effects on their formation and dissociation are not expected (Melander & Saunders, 1980). Sizable N isotope effects occur only on steps involving chemical reaction of the substrate. Hence β_1 , β_2 , β_3 and β_4 should have values very close to 1.000, and eqn 5 simplifies to

$$\beta_{\text{obs}} = \frac{\beta_5(1 - P)(1 - Q) + Q}{1 - P + QP} \quad (6)$$

Under steady-state conditions, influx - efflux = v , or $1 - \text{efflux}/\text{influx} = v/\text{influx}$. Since influx is equal to $k_1[\text{NO}_3^-(\text{out})]$, the following relation holds (eqn 4)

$$1 - \frac{\text{efflux}}{\text{influx}} = \frac{PQ}{1 - P + PQ} \quad (7)$$

Solving this expression for P and Q , in terms of efflux/influx, gives

$$P = \frac{1 - \frac{\text{efflux}}{\text{influx}}}{1 - \frac{\text{efflux}}{\text{influx}}(1 - Q)} \text{ and} \quad (8)$$

$$Q = \frac{\left(1 - \frac{\text{efflux}}{\text{influx}}\right)(1 - P)}{\frac{\text{efflux}}{\text{influx}}P} \quad (9)$$

(The terms P , Q and efflux/influx are all constrained, by definition, to lie between zero and one. Q does not approach infinity as P approaches zero, because in this limit, efflux/influx approaches one.)

Substituting these expressions into eqn 6 provides expressions for β_{obs} in terms of the ratio of efflux to influx, β_5 , and either P or Q .

$$\beta_{\text{obs}} = 1 + \frac{\text{efflux}}{\text{influx}}(1 - Q)(\beta_5 - 1) \text{ and} \quad (10)$$

$$\beta_{\text{obs}} = \beta_5 - \frac{1}{P}\left(1 - \frac{\text{efflux}}{\text{influx}}\right)(\beta_5 - 1) \quad (11)$$

Fig. 2. Expected functional form of the relationship between the relative rate of net NO_3^- uptake (rate in treated samples relative to rate in control samples) and β_{obs} , calculated from eqns 10 and 11, as described in the text. $\circ$, Treatment 1 – only k_5 affected; $\square$, treatment 2 – only $k_3[\text{NR}]$ affected; $\triangle$, treatment 3 – only k_1 affected.

Note that if either P or Q is constant, the relationship between efflux and β_{obs} is linear, provided that β_5 is constant. P , Q and β_5 are unknown. Therefore, the ratio of efflux to influx cannot be determined directly by experimental measurement of β_{obs} . However, the effect of a change in v on β_{obs} can be used to determine whether the change in v resulted from a change in influx or metabolism. A decrease in the net rate of NO_3^- uptake can result from a decrease in k_1 (because, for example, of a decrease in ATP concentration which is subsumed in k_1), $k_3[\text{NR}]$ (because of a decrease in NR concentration) or k_5 (which might happen if NR is reduced after binding with N_2 and if the concentration of reduced ferredoxin is decreased). It can also result from some combination of these, but for the moment we will consider the effect of these changes on efflux/influx and β_{obs} individually.

(1). As discussed above, if the decrease in net NO_3^- uptake rate is caused by a decrease in influx ($k_1[\text{NO}_3^-]_{\text{out}}$) alone (i.e., with no concomitant decrease in $k_3[\text{NR}]$ or k_5), neither partition factor (P nor Q) will be affected. Therefore, the decrease in influx will result in a proportional decrease in efflux (eqn 7). Since both efflux/influx and Q remain constant under this condition, β_{obs} will not be affected (eqn 10).

(2). If the concentration of free NR is lowered, Q should not be affected, but P , will be decreased. As a consequence, both efflux/influx (eqn 7) and β_{obs} will increase, with the relationship between β_{obs} and the net rate of NO_3^- uptake being linear (eqn 10).

(3). If k_5 is decreased, Q will decrease, with a consequent increase in both efflux/influx (eqn 7) and β_{obs} (eqn 11).

These concepts are illustrated in Fig. 2 which shows the relationship between β_{obs} and the net uptake rate calculated from eqns 10 or 11. The calculations were based on arbitrarily assigned values of β_5 and P or Q .

Table 1. Values of k (exponential growth rate constant) and β_{obs} (observed isotopic fractionation factor) during growth of *Synechococcus*

Synechococcus sp. was grown under standard conditions (BG-11 medium containing $1.8 \mu\text{M}$ -molybdate, bubbled with 5% CO_2 in air). Experiments I, II and III were replicate experiments in which the initial NO_3^- concentration was 2 mM. A higher initial concentration (5 mM) was used in experiments IV and V, which are duplicate experiments.

Experiment	Initial NO_3^- concn (mM)	k (h^{-1})	No. of Sampling times	β_{obs} (Mean $\pm$ SE)
I	2.0	0.043	5	1.0020 ± 0.0009
II	2.0	0.035	4	1.0022 ± 0.0009
III	2.0	0.037	6	1.0014 ± 0.0003
IV	5.0	0.038	8	0.9990 ± 0.0006
V	5.0	0.032	4	0.9979 ± 0.0008

The functional form of the relationships of Fig. 2 is independent of the values assigned.

In the discussion above, we examined the consequences of decreasing either k_1 , $k_3[\text{NR}]$ or k_5 , in the absence of an effect of changing any of these on the others. In fact, any treatment which causes a decrease in influx may affect the partition factors P and Q . For example, the concentration of free NR would increase as influx decreases because of the decrease in concentration of intracellular NO_3^- , and k_5 might also increase, if reduced ferredoxin enters the reaction after NO_3^- binds with NR, because of the decrease in concentration of the intermediate of step 5 ($\text{NR} \cdot \text{NO}_3^-$). In either case, P and Q would increase, with a consequent decrease in efflux/influx (eqn 7) and β_{obs} (eqns 10 and 11). Therefore, the effect of decreasing influx will have either no effect on β_{obs} , nor will it decrease its value. In either case, the effect of altering influx on β_{obs} should be distinguishable from the effect of changing the rate of NO_3^- reduction.

Growth curves

To determine the isotope effect associated with net NO_3^- uptake under standard conditions, and whether the magnitude of this isotope effect varied during growth, cultures supplied with NaNO_3 were sampled at intervals during growth. Triplicate experiments (experiments I, II and III, Table 1) were done with an initial concentration of 2 mM- NO_3^- and duplicate experiments (experiments IV and V, Table 1) with an initial concentration of 5 mM- NO_3^- . There was no intended difference among replicate experiments. Growth rate constants (k values) were determined from the best fit of spectrophotometric data to exponential functions and β_{obs} was determined as described in Methods. Results are summarized in Table 1. Growth was exponential; that is, k was constant

(independent of sampling time) within individual experiments. However, from experiment to experiment k varied between 0.032 and 0.043 h⁻¹, with no significant differences between the two treatments (2 mM- and 5 mM-NO₃⁻). β_{obs} was also independent of sampling time within individual experiments and was very close to 1.000 in all experiments, ranging from 0.998 to 1.002. This indicates that, under standard culture conditions, any isotope effects associated with catalytic steps were not vigorously expressed in β_{obs} . Thus, under standard conditions, the rate-limiting step preceded any isotopically sensitive step. There was no correlation between k and β_{obs} , suggesting that whatever unknown variable causing differences among replicate experiments in the net rate of N utilization did not significantly affect the ratio of efflux to influx of NO₃⁻. However, there appeared to be a significant difference in isotopic fractionation between experiments with 2 mM- and 5 mM-NO₃⁻. While the magnitude of β_{obs} with both concentrations was very close to 1.000, a comparison of all data, pooled within each concentration, showed the difference of about 3‰ to be significant at the 99.9% confidence interval. We have no interpretation of this small concentration effect on β_{obs} .

Experimental manipulation designed to alter the rate of assimilation relative to efflux

Substitution of tungstate for molybdate in the growth medium of NO₃⁻-utilizing organisms results in formation of inactive NR (Lara *et al.*, 1987). A wild-type strain of *Anacystis nidulans* (*Synechococcus*) R2, partially inhibited with varying concentrations of tungstate (in the absence of added molybdate), showed decreasing net NO₃⁻ uptake rates with decreasing NR activity (Madueño *et al.*, 1988). Variable inhibition of net NO₃⁻ uptake with tungstate offers the opportunity to test one of the predictions of Fig. 2. Tungstate treatment should have no effect on influx. If reductant concentration is likewise unaffected, the only result of tungstate treatment should be to decrease the value of P ($=k_3[\text{NR}]/\{k_2 + k_3[\text{NR}]\}$), thus decreasing overall velocity, v (eqn 4), and increasing the ratio of efflux to influx (eqn 7). Our expectation was that β_{obs} should increase with increasing tungstate concentration and decreasing net NO₃⁻ uptake rate (eqn 10).

Tungstate concentration in the growth medium was varied between 0 and 0.2 mM in the presence of 0.18 µM-molybdate (the usual molybdate concentration was 1.8 µM). Samples were collected when the organisms had consumed about half the NO₃⁻ initially present (2 mM), estimated by spectrophotometric measurement of growth.

Fig. 3(a) shows the exponential growth rate constant, k , as a function of tungstate concentration. The value of k

Fig. 3. (a) Exponential growth rate constant, k , as a function of tungstate concentration; (b) variation of β_{obs} with tungstate concentration; (c) variation of β_{obs} with k .

declined from 0.040 to 0.013 h⁻¹ (corresponding to an increase in doubling time from 17.3 to 53.3 h) over the concentration range of tungstate used, a decline of about 70%. The relationship between tungstate concentration and k was linear ($r = 0.98$, $p < 0.005$). With no added tungstate, the value of k for a parallel experiment with the usual molybdate concentration (1.8 µM) was not significantly different from the value of k with 0.18 µM molybdate.

Over the same tungstate concentration range, β_{obs} increased from 1.0023 to 1.0132 (Fig. 3b), as expected from the anticipated increase in the ratio of efflux to influx, with increasing tungstate.

Provided that Q was unaffected by tungstate treatment, it was expected that there should be a linear decrease in β_{obs} with k (Fig. 2). This expectation was borne out (Fig. 3c) by the high correlation coefficient for

the relationship between β_{obs} and k ($r = 0.97$, $P < 0.02$). The y intercept of the linear regression shown on this panel corresponds to a value of v of zero. When v approaches zero, efflux approaches influx under steady-state conditions; that is, the ratio of efflux to influx approaches 1. Thus the value of β_{obs} approaches $1 + (1 - Q)(\beta_5 - 1)$ (eqn 10) when v approaches zero. The data of Fig. 3 allow us to estimate this value at 1.0197. When the ratio of efflux to influx approaches zero, the value of β_{obs} approaches 1.000, since in this limit, the non-fractionating, rate-limiting step, namely influx, determines the overall rate. Since under the conditions of this experiment the relationship between β_{obs} and k was linear, it is possible to calculate the ratio of efflux to influx for any experimentally determined value of β_{obs} from a linear interpolation between the two limits, where efflux/influx approaches zero or one, using eqn 10. For example, the value of β_{obs} in the absence of tungstate, and in the presence of $0.18 \mu\text{M}$ -molybdate, calculated from the regression, is 1.0037. From this value, we calculate that the ratio of efflux to influx is about 0.19. That is, in the absence of tungstate, more than 80% of NO_3^- entering the cell undergoes reduction with less than 20% re-entering the medium from the cell, even when the molybdate concentration was decreased by a factor of 10 below the normal concentration. With the normal molybdate concentration ($1.8 \mu\text{M}$), the value of β_{obs} was 1.0003, implying that more than 99% of the NO_3^- entering the cell undergoes reduction.

The small difference in β_{obs} for the two molybdate concentrations (1.8 and $0.18 \mu\text{M}$) (Fig. 3b) is consistent with the NR concentration being limiting at the lower molybdate concentration. In this case, one might have expected a lower value of k for the lower molybdate concentration. This was not observed (Fig. 3a). However, there was no correlation between k and β_{obs} in replicate experiments (Table 1), suggesting an unknown variable causing differences in k but which does not affect the ratio of efflux to influx. It seems possible that a difference in k caused by NR concentration might be offset by such an unknown variable.

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