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Mechanism of sodium/nitrate symport in *Anacystis nidulans* R2

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Abstract

A kinetic study of sodium-dependent nitrate transport in the cyanobacterium *Anacystis nidulans* R2 has been performed by following intracellular accumulation of nitrate in intact cells of the mutant strain FM6, lacking nitrate reductase activity and unable, therefore, to reduce the transported nitrate. Initial transport rates were determined at different external fixed sodium and varying nitrate concentrations and, conversely, at different fixed nitrate and varying sodium concentrations. The resulting kinetic pattern has the best fit to a reaction mechanism model in which sodium nitrate is the substrate for the transporter and sodium behaves additionally as a non-essential activator of the system. Half-saturation constants for the substrate sodium nitrate (1.6 ± 0.2 μM) and the activator sodium (0.36 ± 0.04 mM) have been calculated. The operation of such a sodium/nitrate symport system, driven by the energy of the $\Delta\tilde{\mu}_{Na^+}$ across the plasma membrane, provides the basis for energy coupling between uphill nitrate and downhill sodium transport into *Anacystis* cells.

Key words: Nitrate transport; Sodium bioenergetics; Symport mechanism; Cyanobacterium

1. Introduction

Nitrate transport into the cell is the rate-limiting step of nitrate assimilation in cyanobacteria. In the unicellular cyanobacterium *Anacystis* (*Synechococcus*) the active transport of nitrate is subjected to a stringent control of activity by products of both ammonium and CO_2 assimilation [1], as well as to repression by ammonium [2].

Recently, a mutant strain of *Anacystis nidulans* R2 named FM6, which lacks nitrate reductase activity [3] and is therefore unable to reduce the transported nitrate, has been used for kinetic and bioenergetic analysis of nitrate transport [4]. The FM6 mutant has a nitrate transport capacity analogous to those of the wild-type strain R2 [4] and of the closely related species *A. nidulans* (*Synechococcus leopoliensis* 1402-1, [1,2]). Nitrate transport in FM6 cells is also subject to nutritional repression by ammonium, as well as to feedback inhibition by ammonium assimilation products [5]. These analogies allow us to derive essential reliable information on the mechanism of nitrate transport in *Anacystis* from studies performed in this mutant strain.

Under illumination and optimal conditions, cells of the FM6 mutant strain rapidly accumulated intracellular nitrate against the nitrate electrochemical gradient, with a ΔG of about + 20 kJ/mol of nitrate at steady state [4]. The process also exhibited a Q_{10} value of 3 [4], characteristic of carrier-mediated transport systems [6]. Nitrate transport showed a strong and selective requirement for Na^+ and was highly sensitive to monensin, an ionophore which relaxes the sodium electrochemical gradient [4]. These observations indicate that nitrate transport activity relies on the maintenance of the $\Delta\tilde{\mu}_{Na^+}$ across the plasma membrane, which might represent the immediate source of energy for active nitrate transport [4,7]. Interestingly, under normal growing conditions (pH 8.0–8.5 and 15–20 mM Na^+), the $\Delta\tilde{\mu}_{H^+}$ across the plasma membrane of *Anacystis* is near zero and the $\Delta\tilde{\mu}_{Na^+}$ amounts to about -10 kJ/mol [8]. The $\Delta\tilde{\mu}_{Na^+}$ would be the driving force for secondary solute transport [7,9].

At saturating sodium concentrations, nitrate transport exhibited complex saturation kinetics with respect to external nitrate, with apparent substrate inhibition

Abbreviations: Tricine, (*N*-tris[hydroxymethyl])methylglycine; Tris, tris[hydroxymethyl]aminomethane.

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at nitrate concentrations above 25 μM. The Na^+ requirement and the apparent substrate inhibition of nitrate transport suggested the operation of a symport of nitrate and sodium [4,7]. In this work, a more detailed kinetic study has been undertaken to test the occurrence of a sodium/nitrate symport and further investigate the mechanism of nitrate transport in *Anacystis*.

2. Materials and methods

Anacystis nidulans R2 (*Synechococcus elongatus* PCC 7942) strain FM6, which lacks nitrate reductase activity, was grown photoautrotophically at 40°C as previously described [3] with 7.5 mM $(NH_4)_2SO_4$ as the nitrogen source. Nitrate transport was expressed by transferring the cells to a medium containing 4 mM KNO_2 as the nitrogen source, since nitrite is an alternative substrate for the nitrate transport system in *Anacystis* [4]. After 18 h, the cells were harvested by centrifugation, washed and resuspended in the buffer used for the nitrate transport assay [4]. Chlorophyll *a* was determined after methanol extraction [10].

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. [11], with the following modifications. The nitrate transport assay was carried out at 40°C in air-opened conical flasks with continous shaking and illumination. The assay medium (5 ml) contained 25 mM Tricine/90 mM Tris buffer (pH 8.3 at 40°C), an amount of cells equivalent to 33 μg of chlorophyll *a*/ml and variable concentrations of NaCl and of HNO_3. The assay was started by simultaneous addition of nitrate and illumination. At 0.5 min intervals, aliquots (0.3 ml) were withdrawn and rapidly transferred to 0.4 ml polyethylene microcentrifuge tubes over a silicone-oil layer (0.08 ml of a 2:1 (v/v) mixture of Versilube F50 (Serva) and silicone 14615–3 (Aldrich)), which was underlaid by 0.02 ml of 20 mM sulphamic (amidosulphuric) acid in 2 M H_3PO_4. After rapid centrifugation (10000 $\times g$, 1 min) in a Beckman Microfuge 11, nitrate was analyzed in aliquots of the acid cell lysate by ion-exchange high presure liquid chromatography followed by UV detection of nitrate at 210 nm [11]. Intracellular nitrate concentrations have been calculated for an aqueous internal volume for *A. nidulans* cells of 100 μl/mg of chlorophyll *a* [12]. Correction for extracellular nitrate associated with the cells as they sediment through the silicone oil was done by substracting the nitrate present in the acid-cell lysate at time zero. Initial rates of nitrate transport were taken as the mean velocity during the linear initial intracellular nitrate accumulation period (1 or 2 min at low or high sodium, respectively), and related to the corresponding mean substrate concentration ($[S] = 1/2\ ([S]_o + [S])$), to correct for the nitrate transported over this period [4,13].

3. Results and discussion

The strict Na^+ requirement of nitrate transport in *Anacystis* together with its apparent inhibition by excess nitrate observed in our previous study suggested the operation of a nitrate/sodium symport system [4]. Depending on the reaction mechanism, bisubstrate enzymes [13–16] and carriers [17–19] can exhibit apparent substrate inhibition with respect to either or both substrates, this behaviour being especially manifest in systems involving metal ions.

Intracellular nitrate accumulation by FM6 cells was linear for at least 1–2 min at any nitrate and sodium concentrations tested (data not shown [4]). When initial rates of nitrate transport were determined as a function of external nitrate concentrations at different fixed external Na^+ concentrations, inhibition by excess nitrate always appeared (Fig. 1). The shape of the velocity curves was similar, i.e., initially hyperbolic until reaching a peak velocity, and declining thereafter to stabilize for supraoptimal nitrate concentrations at rate values below the maximum. Interestingly, as the concentration of external Na^+ was increased, the observed peak velocity was progresively higher, and it was reached at increasingly higher external nitrate concentrations. Thus, for 0.5 mM Na^+, the peak velocity was reached at about 5 μM external nitrate. Increasing the external Na^+ concentration to 1 mM resulted in a shift of the peak velocity position to about 10 μM nitrate. When external Na^+ was 10 mM, the peak velocity position shifted to about 25 μM nitrate (Fig. 1). In all cases, for a given nitrate concentration, the nitrate transport rate was dependent on the concentration of external Na^+. For supraoptimal nitrate concentrations, nitrate transport rate stabilized at about 15 μM/min for 0.5 mM Na^+, 35 μM/min for 1 mM Na^+, and 45 μM/min for 10 mM Na^+.

When initial rates of nitrate transport were determined as a function of external Na^+ concentrations at different fixed external nitrate concentrations, saturation kinetics for Na^+ resulted in all cases (Fig. 2), validating our previous proposal of a sodium-nitrate symport. This set of kinetics was also complex. Thus, at low nitrate (10 μM), nitrate transport exhibited abrupt saturation by Na^+, and the velocity stabilized at about 75 μM/min above 0.1 mM Na^+. At 20 μM nitrate, two concave profiles appeared in the velocity curve. After a first apparent saturation by Na^+ at about 0.1 mM, with a similar rate than above, the velocity raised again as Na^+ increased and Na^+-saturation was reached at about 0.5 mM. For higher fixed nitrate (30 μM), the

first concave phase was again evident, the interface between the concavities of the curve softened, and saturation appeared at a still higher Na^+ concentration (above 0.8 mM) with a rate also higher (Fig. 2). Interestingly, nitrate transport was not inhibited by excess Na^+ at the concentrations assayed.

A carrier-mediated cotransport can be percieved analogous to an enzyme bi-bi reaction in which the transportable species are substrates when they are on one side of the membrane and products when they are on the other side [17–19]. The kinetic behaviour of nitrate transport in *A. nidulans* R2 FM6 cells fits into a model developed by London and Steck [14] for enzyme reactions with interaction between a substrate (reactant) and a metal ion modifier, such as the prototype reaction involving ATP (reactant) and Mg^{2+} (modifier), in which the true substrate is the Mg-ATP^{2-} complex and the metal ion modifier can also bind to the enzyme at a specific site. The general model assumes that the enzyme exists in an unmodified (less active) and a modified (more active) form, and that the metal ion modifier and the reactant combine with each other and with the enzyme to form an enzyme-modifier-reactant complex. Criteria for distinguishing effects of the modifier and the reactant include the shape of the velocity curves, the occurrence of apparent substrate inhibition by excess reactant and/or modifier, and the shifting in the position of the peak velocity [13,14]. When these criteria are applied, the features of the observed nitrate transport kinetics have the best fit into a particular case of the general model in which the true substrate is the reactant-modifier complex, and the modifier acts additionally as a non-essential activator [13,14]. According to this, sodium nitrate would be the true substrate of the nitrate carrier, and Na^+ would act as a non-essential activator. As schematically depicted in Fig. 3, the substrate sodium nitrate might bind to the active site of either

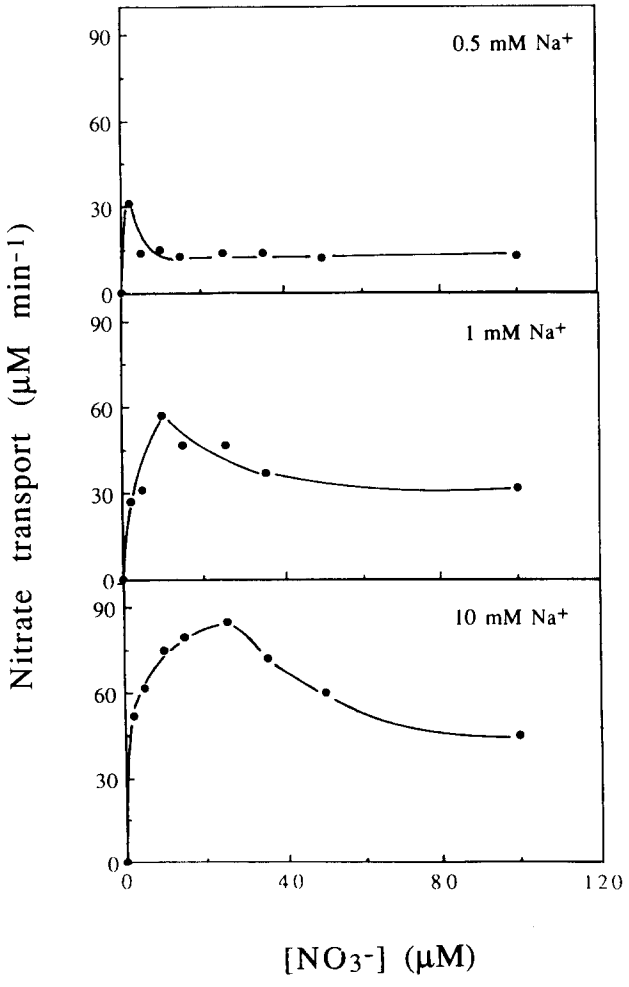

Fig. 1. Initial rates of nitrate transport as a function of nitrate concentrations at different fixed sodium concentrations in intact cells of *Anacystis nidulans* R2 FM6 mutant. A representative experiment from a minimum of three similar replicates.

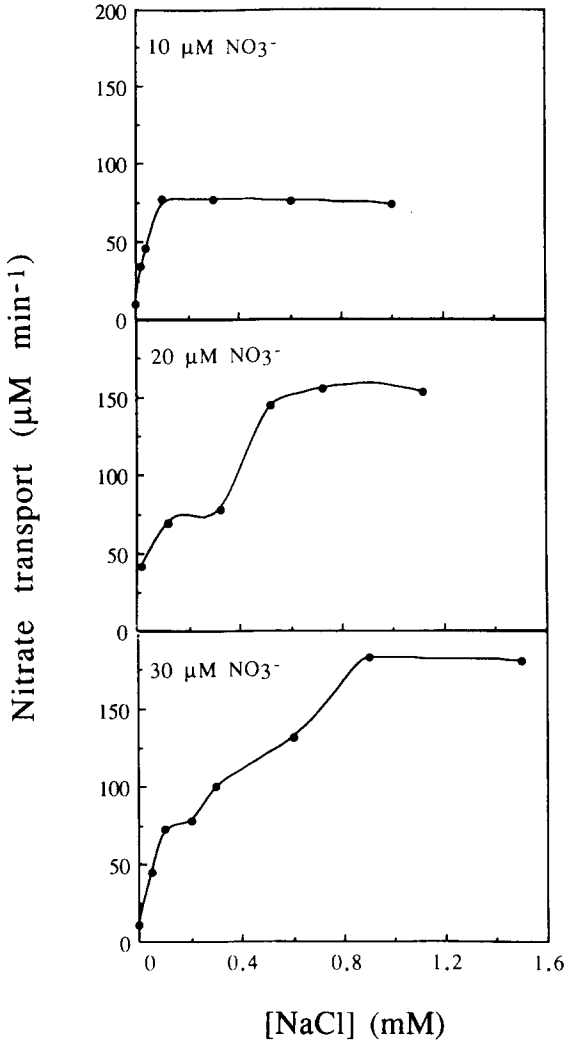

Fig. 2. Initial rates of nitrate transport as a function of sodium concentrations at different fixed nitrate concentrations in intact cells of *A. nidulans* R2 FM6 mutant. A representative experiment from a minimum of three similar replicates.

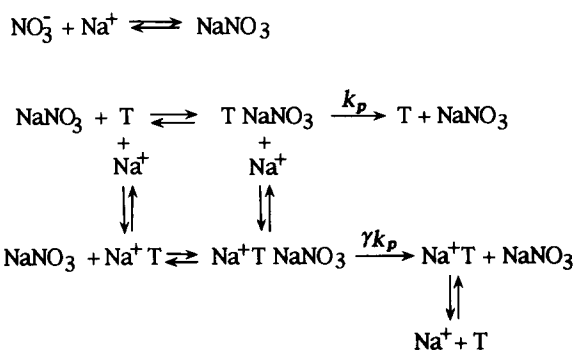

Fig. 3. Reaction mechanism model for cotransport of nitrate and sodium in *A. nidulans*. The velocity equation would be given by Eq. 1. Explanation in the text.

the transporter (T) or the transporter-activator complex (Na^+-T), while free sodium ions would bind to a specific activator site on either the transporter or the transporter-substrate complex (T-$NaNO_3$) giving rise to the activated form of the carrier. Both the transporter-substrate and the transporter-substrate-activator (Na^+-T-$NaNO_3$) complexes are susceptible of translocation, although the rate constant in the latter case is higher than in the former. The corresponding velocity equation [13,14] is obtained from:

$$v = k_p\,[\text{T-NaNO}_3] + \gamma\, k_p\,[\text{Na}^+\text{-T-NaNO}_3] \qquad (1)$$

being $\gamma > 1$

Thus, in Fig. 1, at low nitrate concentrations, the observed velocity would result primarily from the operation of the activated transporter, the relative proportion of which depends on the external Na^+ concentration. After reaching the peak velocity, the system becomes progresively inhibited by free nitrate [13]. Decreasing the fixed Na^+ concentration results in declining peak velocity values and in a shift of their position to lower nitrate concentrations, as less sodium nitrate and more free nitrate are present for a given total nitrate concentration. The K_s value of the activator-transporter-substrate complex (1.6 ± 0.2 μM sodium nitrate) is determined from the hyperbolic part of the velocity curve at saturating Na^+ concentration (10 mM, see below). Complex kinetics in Fig. 2 can also be explained by the model in Fig. 3. At low sodium concentrations, the observed velocity results primarily from the operation of the non-activated transporter. When the concentration of nitrate is low (10 μM), the system is limited by substrate availability and the curve is concave. When the nitrate concentration is higher, after the initial phase of non-activated transport, activation by sodium is showed by a superimposed second concave profile, as the relative proportion of the activated carrier increases [13,14].

This reaction mechanism model (Fig. 3) for cotransport of nitrate and sodium in *A. nidulans* predicts a minimum stoichiometry of one sodium per nitrate transported when the non-activated form of the transporter operates, and a maximum stoichiometry of two sodium ions per nitrate transported when the activated form of the transporter is operative and the activator Na^+ is also released from the transporter inside the cell in each transport cycle.

Hill plots of data such as those in Fig. 1 for suboptimal nitrate concentrations yielded straight lines with a slope $n = 1.05 \pm 0.03$ (not shown), indicating that only one sodium nitrate binds to the activated form of the transporter, and an $[S]_{0.5}$ value of 1.6 ± 0.2 μM sodium nitrate. This value is identical to the K_s for sodium nitrate calculated above, and very close to the previously estimated K_s value of 1 μM [4]. Interestingly, Hill plots of data such as those in Fig. 2 were non-linear, with a slope of $n < 1$ at low sodium concentrations, an inflexion point at the $[Na^+]_{0.5}$ value (0.36 ± 0.04 mM), and a slope $n = 3.2 \pm 0.2$ for higher Na^+ concentrations (not shown). Apparent negative cooperativity with respect to the activator can arise from the kinetic model of Fig. 3, without necesarily meaning multiple interacting sites [13,14]. The high n value observed at high Na^+ concentrations suggests, however, the posibility of more than one activator site in the transporter. The nitrate transporter might bind two sodium ions at activator sites in addition to the one forming the sodium nitrate complex, which binds to the substrate site. More detailed kinetic studies are needed to assess the actual number of Na^+ ions involved in the activation of the nitrate transporter.

According to this, a kinetic scheme for sodium/nitrate symport can be proposed (Fig. 4). One substrate molecule (S) and/or a number n, still to be assessed, of activator sodium ions (A) can bind to the free carrier

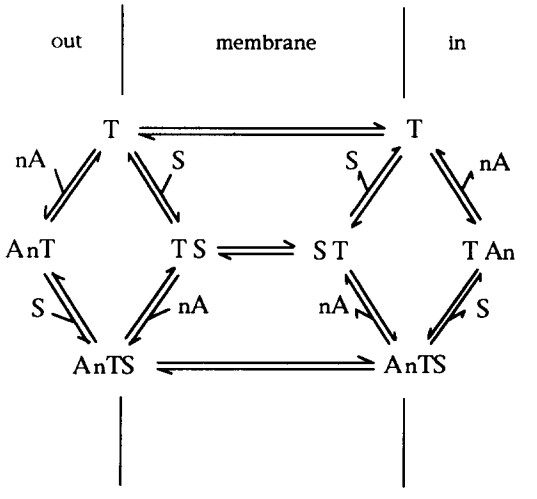

Fig. 4. Kinetic scheme for nitrate/sodium symport across the plasma membrane in *A. nidulans*.

(T). The binary transporter-substrate (TS) complex (non-activated form) as well as the transporter-substrate-activator(s) (A_nTS) complex (activated form) would be mobile (i.e. the substrate and activator(s) binding sites can move from exposure on one side of the membrane to exposure on the other [20]). This would allow the translocation across the plasma membrane of sodium nitrate and, additionally, of extra sodium ions when the activated form of the transporter operates, coupling the endergonic entrance of nitrate to the exergonic entrance of sodium. Best coupling is achieved if the transporter-activator(s) (TA_n) complex is immobile [20] and unable to facilitate diffusion of sodium. Upon release of the transported species, the free carrier would reorientate, completing the transport cycle. It is oportune to note that the external sodium concentration will determine the relative proportion of the two forms, activated and non-activated, of the transporter. On the other hand, the internal sodium concentration will determine the release of the sodium activator(s). The magnitude of the $\Delta\tilde{\mu}_{Na^+}$ across the plasma membrane would, therefore, determine not only the rate of transport but also the sodium/nitrate stoichiometry, conforming a flexible coupling system able to adapt to the energetic conditions of the cell.

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