A High Affinity Fungal Nitrate Carrier with Two Transport Mechanisms*

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We have expressed the CRNA high affinity nitrate transporter from Emericella (Aspergillus) nidulans in Xenopus oocytes and used electrophysiology to study its properties. This method was used because there are no convenient radiolabeled substrates for the transporter. Oocytes injected with crnA mRNA showed nitrate-, nitrite-, and chloride-dependent currents. Although the gene was originally identified by chlorate selection there was no evidence for transport of this anion. The gene selection is explained by the high affinity of the transporter for chlorite, and the fact that this ion contaminates solutions of chlorate. The pH-dependence of the anion-elicited currents was consistent with H−-coupled mechanism of transport. At any given voltage, currents showed hyperbolic kinetics with respect to extra-
membrane voltage and external pH (6). Nitrate uptake had complex kinetics in E. nidulans but the K_m was estimated as 200 μM (7).

The crnA mutants of E. nidulans were first isolated as a class of chlorate-resistant strains that were able to utilize nitrate as the sole nitrogen source (8). The crnA mutants were subsequently found to be defective in nitrate uptake at the conidiospore and young mycelial stages (7). The crnA gene has been cloned (9) and belongs to the Major Facilitator superfamily of membrane transporters, being in the same family (nitrate-nitrite porter, NNP) as NarK, the nitrite efflux system from Escherichia coli and the NRT2 high affinity nitrate transporters from algae and higher plants (10). A related nitrate transporter from the yeast Hansenula polymorpha has also been isolated (11). Genes encoding high affinity nitrate transporters from the alga, Chlamydomonas reinhardtii have been isolated and complementation of mutant strains has shown that two genes (Nar2 and either NRT2;1 or NRT2;2) are necessary for a functional nitrate transport system (12). In Chlamydomonas, nitrate and nitrite are transported by different specific transport systems and also by a nonspecific transporter (13). The injection of a mixture of Nar2 and Nrt2;1 mRNA into Xenopus oocytes gave nitrate transporter activity, but Nar2 mRNA was also toxic to these cells (14). Recently, evidence for a mammalian H+/NO_3− cotransport mechanism has also been described (15).

To date there are no reports of the detailed characterization of high affinity nitrate transporters in a heterologous expression system, although members of the NRT1 family of low affinity transporters have been studied in Xenopus oocytes (16, 17). As in Chlamydomonas, a high affinity nitrate transport system requires two gene products; we tested if crnA, the first nitrate transporter gene to be isolated, encodes a fully functional uptake system. This is important because many homologs of the NNP family have recently been cloned by sequence homology to crnA (18), but the function of these cannot

Nitrate is an important nitrogen source for many organisms ranging from bacteria and cyanobacteria to fungi and plants. Nitrate carriers for transport across the plasma membrane must be present in many different cell types. In fungi, nitrate transport has been demonstrated in Penicillium chrysogenum (1) and Neurospora crassa (2), and the presence of H+/nitrate symport activity was shown in the plasma membrane of cells of Candida utilis (3) and Emericella (Aspergillus) nidulans (4). In N. crassa, the nitrate transport system was induced by the external supply of NO_3− and the K_m was 0.25 mM, whereas the nitrite system had a K_m of 86 μM (5). Electrophysiological characterization of nitrate transport in intact hyphae of N. crassa showed that high affinity transport was sensitive to both membrane voltage and external pH (6). Nitrate uptake had complex kinetics in E. nidulans but the K_m was estimated as 200 μM (7).

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easily be demonstrated. The plant family members are of fundamental importance to plant biology and agriculture because nitrogen supply is the main factor limiting growth and yield of crops. Functional characterization of the NNPps is hindered by the lack of convenient transceivers for the transported substrates. 

The pH was adjusted by the addition of 5M NaOH solution. Experiments were performed in a saline containing (in mM) 116 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.2. For experiments that had been vigorously aerated for 1 h. In all experiments, the oocytes were allowed to adjust for at least 5 min after changing the external pH before any treatments were applied.

**RESULTS**

**Substrate Specificity of CRNA**—To obtain the *crnA* mRNA necessary for oocyte expression studies, we needed a full-length *crnA* cDNA sequence in a suitable transcription vector. However, the only *crnA* cDNA clone available (pSTA1500, Ref. 9) was incomplete at the 5′-end, whereas the cloned *crnA* gene (in pSTA4) was also unsuitable for our purposes because it contained three introns (9). We therefore used RT-PCR to generate the missing 5′ part of the cDNA sequence and combined this with a segment of the pSTA1500 insert to create a full-length *crnA* cDNA clone, pCRNA5, which was then fully sequenced to establish its authenticity (see "Experimental Procedures"). Because of an error in assigning the precise location of one of the introns, *crnA* was originally reported to encode a polypeptide of 483 amino acids with 10 transmembrane domains (9). The *crnA* sequence in pCRNA5 was confirmed as encoding a polypeptide of 507 amino acids and 12 predicted transmembrane domains (see GenBank™/EBI accession no. U34382).

The anion-elicted currents were measured in oocytes injected with the *crnA* mRNA. The experiment was performed using the two-electrode voltage clamp technique. Oocytes injected with *crnA* mRNA for the truncated form of CRNA did not show any nitrate-elicted currents (data not shown). Fig. 1A shows the I-V difference curves obtained for membrane voltages (*V*ₘ) between 0 and 160 mV for oocytes injected with full-length *crnA* mRNA. Subtracting the I-V relationship obtained in the absence of substrate from that obtained in its presence generated these curves. Both nitrate and nitrite (applied as the sodium salt at a concentration of 0.1 mM and at pH7) elicited currents in oocytes, which had been vigorously aerated for 1 h. In all experiments, the oocytes were allowed to adjust for at least 5 min after changing the external pH before any treatments were applied.
dependence of the NO$_3^-$ which the sodium was replaced with choline tested the sodium-concentration. Replacing the bathing saline with a solution in significant current when treated with any of these ions at the same concentration was varied from 1 to 200 $\text{mM}$ to generate a family of I-V curves. The steady-state currents were measured as a function of voltage for each NO$_3^-$ concentration. These nitrate-elicited currents became larger at more negative membrane potentials and appeared to saturate at an external NO$_3^-$ concentration between 40 and 60 $\mu\text{M}$.

Fig. 2A shows the relationship between the nitrate transport activity of CRNA and the external NO$_3^-$ concentration from 1 to 150 $\mu\text{M}$ at pH$_o$ 7 for seven different membrane voltages. These results confirmed that CRNA was a high affinity NO$_3^-$ transporter, with the transport activity saturating around 40 $\mu\text{M}$, but the data only fit a Michaelis-Menten function at the lower membrane voltages. A similar pattern was observed for the other anion substrates, and an example for ClO$_2^-$ but with no fitted line is shown in Fig. 2B.

Fig. 1. $I$-$V$ curves showing the anion substrate specificity of a crnA mRNA-injected oocyte. Difference currents were generated by treating the oocyte with sodium salts of nitrate (○), nitrite (▲), freshly prepared chloride (▼), and 28-day-old chloride solution (□) all at the same concentration (100 $\mu\text{M}$) at pH$_o$ 7 in nitrate-free saline (A). The voltage-sensitivity (B) of a family of different sodium nitrate concentrations (1 to 200 $\mu\text{M}$) was obtained. Each data set is an $I$-$V$ curve obtained from the same oocyte treated with nitrate at concentrations of 2.5 (○), 5 (□), 10 (▲), 20 (▼), 40 (■), 60 (▲), 150 (○), and 200 (□) $\mu\text{M}$. The $I$-$V$ curves were determined as described previously (17).

Voltage-dependence of $i_{\text{max}}$ and $K_m$ for Protons—Steady-state NO$_3^-$-elicited currents were measured as a function of voltage and pH$_o$. At a fixed external NO$_3^-$ concentration (200 $\mu\text{M}$) and at any given voltage the data from the $I$-$V$ difference curve fit a single Michaelis-Menten function (Fig. 3A). Fig. 3B shows the voltage-independence of the $K_m^H$ values calculated from these fits. The values for $K_m^H$ were also voltage-dependent, changing from 0.14 $\mu\text{M}$ (pH 6.85) at 0 mV to 0.004 $\mu\text{M}$ (pH 8.4) at -180 mV (Fig. 3B). The $i_{\text{max}}^H$ values also obtained from these lines
were voltage-dependent, increasing from −26 nA at 0 mV to −63 nA at −180 mV (data not shown).

*Inhibition by Anion Substrates*—When an oocyte expressing CRNA was treated with nitrate concentrations from 1 μM to 20 mM and the concentration was plotted on a log scale, the substrate inhibition became very clear (Fig. 4). At more negative voltages and at NO$_3^−$ concentrations larger than 80 μM, the current mediated by the transporter decreased, showing an inhibition of transport activity by NO$_3^−$. For example, at −150 mV the current in 20 mM NO$_3^−$ was decreased by 50% when compared with that obtained at 100 μM NO$_3^−$. Whereas the equivalent percentage inhibition at −70 mV is 20%, and at −30 mV, there was no inhibition by higher concentrations of nitrate. The data shown in Fig. 4 could be described by a more complicated model that included two additive Michaelis-Menten functions (24). From fits of this model to these data points the $K_m$ and $i_{max}$ values for each function (I and II) could be determined at each membrane voltage (see “Experimental Procedures”). The mean value of $K_m$ was 20.5 μM and was independent of changes in membrane voltage in the range from −160 to −70 mV, whereas at less negative membrane voltages, $K_m$ increased to 88 μM at −30 mV. The rate of decrease of the $K_m$ values was also determined for two other substrates, NO$_2^−$ and ClO$_2^−$. The kinetic parameters were calculated from fits to families of different NO$_3^−$ and ClO$_2^−$ concentrations at pH 7. For both substrates, the parameters $i_{max(NO_2)}$ and $i_{max(ClO_2)}$ were voltage-dependent, increasing as the membrane potential increased more negative, whereas in contrast, both $K_{m(NO_2)}$ and $K_{m(ClO_2)}$ were voltage-independent (data not shown). When compared with nitrate, the values of $K_{m(NO_3)}$ were 3- and 4-fold higher for NO$_2^−$ and ClO$_2^−$, respectively.

**Fig. 4. Voltage dependence of the substrate inhibition of CRNA nitrate transport activity.** Steady-state nitrate-dependent currents at −30 (●), −70 (○), −110 (▼), −150 (▽) mV plotted as a function of external nitrate concentration at pH 7. The lines were fit to the data using two additive Michaelis-Menten functions (Equation 1) using Sigmaplot (see “Experimental Procedures”). The parameter values for the fitted line at −150 mV are $K_m(NO_3)=23$ μM, $i_{max(NO_3)}=126$ μM, $i_{max(NO_2)}=−466$ nA, and $i_{max(ClO_2)}=339$ nA.

The effect of changing pH$_o$ on the nitrate-elicited currents was measured in oocytes injected with crnA mRNA. The nitrate-elicited currents increased as the pH$_o$ was decreased from 8 to 6, but at pH$_o$ 5.5 this effect saturated showing no further increase in current (Fig. 3A). At pH$_o$ 5.5, when the mRNA-injected oocyte was treated with a range of nitrate concentrations, the substrate-inhibition of current was again demonstrated (Fig. 5A). To compare the results obtained at different pH$_o$, the nitrate-elicited currents were normalized to the maximal current obtained and this is shown as the percentage substrate inhibition (Fig. 5B). Fig. 5B shows a comparison of the percentage nitrate inhibition at pH$_o$ 5.5 and 7. The substrate inhibition of current was not effected by a change in pH$_o$ from 7 to 5.5, and similar profiles were obtained at intermediate pH$_o$ values (data not shown).

**Kinetic Model for the Anion-Substrate Inhibition**—Three fundamental observations define the anion-inhibition of CRNA activity. 1) It increases with higher concentrations of anion substrate. 2) It is independent of changes in external pH (over the range 8 to 5.5), and 3) it is voltage dependent, increasing at more negative voltages. This information can be used to build a reaction cycle model of CRNA-mediated transport. A decrease in the inward current can occur by three possible mechanisms, a decrease in the amount of available carrier in the membrane, a decrease in the influx of protons or an increase in anion influx. There are other options that involve changes in efflux (decreased for protons and increased for anions), but these can be discounted because they are thermodynamically unfeasible. The practicability of each of these three mechanisms will be considered.

As the substrate-inhibition of CRNA does not depend on pH$_o$, but increases with the inwardly directed chemical gradient for anions, the most likely mechanism to achieve this result is an increased anion influx. The only mechanism that can account for both of these observations is that nitrate must bind before protons, and some transfer of the anion across the membrane can occur in this form (see Fig. 6). There are usually two recognized steps at which the transmembrane charge transfer can occur. These are the translocation of either the loaded carrier (positive charge) or the unloaded carrier (negative charge). A model involving the binding and translocation of nitrate via a negatively charged empty carrier is thermody-
The NO$_3^-$ transporter CRNA of *E. nidulans* has been heterologously expressed in *Xenopus* oocytes enabling the first electrophysiological characterization of a fungal carrier protein using this expression system. Although there have been *in vivo* electrophysiological studies on high affinity nitrate transport activity in *Neurospora* (6), these measurements could not exclude the possibility that the nitrate transport processes being analyzed were mediated by more than one protein. Oocytes injected with mRNA for a truncated form of the protein did not show any nitrate-elicited currents. These results suggest that this deletion altered the transport activity or the membrane targeting of the protein.

In the green algae *Chlamydomonas*, genetic analysis has established that the *NRT2;1* and *NRT2;2* genes, which are homologous to *crnA* and which encode the membrane proteins responsible for high affinity nitrate and nitrite transport in this organism, require the activity of an additional gene (*Nar2*) to specify functional transport systems (12, 13). Preliminary experiments confirmed that the *Chlamydomonas* *NRT2;1* transporter is not functional when expressed in oocytes, and nitrate transport activity was only measured after co-injection of *Nar2* mRNA (14). Possible roles for *Nar2* include acting as a second subunit for the *NRT2* transporter or an involvement in trafficking of the *NRT2* protein from the endoplasmic reticulum to the plasma membrane. In the oocyte experiments reported here, functional high affinity NO$_3^-$ transport was achieved with a single protein. The ability to obtain functional expression of *crnA* in oocytes without the assistance of a second gene suggests that there may be important differences between the algal and the fungal nitrate transporters, despite the sequence homology between the *crnA* and *NRT2* genes. One striking distinction between the predicted structures of the fungal and the algal nitrate transporters of this family is in the arrangement of the hydrophilic domains within the primary sequence of the proteins. The fungal proteins lack a large C-terminal domain that is found in the algal (and higher plant) NRT2 transporters. Another difference is the lack of a large central loop between transmembrane domains 6 and 7 (10, 18).

The CRNA transporter expressed in oocytes can also transport nitrite and chlorite, but with a lower affinity than nitrate. The *K_m* for NO$_3^-$ of the carrier expressed in oocytes was less than the value obtained for *E. nidulans* cells *in vivo*, for example 200 µM (7). As CRNA has a high affinity for NO$_3^-$, the nitrite-elicited current could have resulted from contamination of the NO$_2^-$ solution with nitrate. To check for this possibility we measured how much nitrate could be present in a solution of nitrite and attempted to favor this oxidation reaction by vigorously aerating a nitrite solution. Even after vigorous aeration for 1 h, less than 1% of the nitrite had been converted to nitrate across the membrane occurs driven by the chemical gradient of nitrate (Fig. 6, dashed line). In other words, nitrate binding to CRNA happens before the proton binding and nitrate slippage through the carrier only occurs at high external concentrations. This step will be independent of the proton gradient because the membrane translocation of nitrate does not require a proton. Although the negative membrane voltage will tend to oppose nitrate translocation into the oocyte, there is a large chemical gradient driving nitrate transport. The nitrate slippage increases at more negative membrane voltages because the proton-coupled charge translocation step increases at more negative voltage providing more unloaded carrier for this process to occur. In support of this aspect of the model the substrate inhibition, relative to the maximal current, increases at more negative voltages; for example, 50% nitrate inhibition at $-150$ mV and 20% at $-70$ mV (see Fig. 3).

**DISCUSSION**

FIG. 5. The effect of pH$_0$ on the nitrate-elicited current of CRNA. A, the steady-state nitrate-dependent currents at $-150$ mV plotted as a function of external nitrate concentration from 1 µM to 10 mM at pH$_0$ 5.5. The line was fit to the data using two additive Michaelis-Menten functions (Equation 1) using Sigmaplot (see “Experimental Procedures”). For this fitted line the kinetic parameters are, $K_m^{\text{NO}_3} = 24$ µM, $i^{\text{NO}_3} = 127$ µM, $i^{\text{NO}_3} = -116$ nA, and $i^{i=\text{NO}_3} = 81$ nA. B, comparison of the substrate inhibition profiles of CRNA at pH 7 and 5.5. These results were obtained using two different oocytes. For comparison the current values have been normalized to the maximum current obtained. The lines are drawn to join points.

**FIG. 6. Reaction kinetic cycle for H$^+$ and NO$_3^-$ cotransport by CRNA.** The main feature of this cycle is that the two processes that can transfer charge across the membrane are the translocation of the loaded CRNA and the proposed “slippage” of the partially loaded carrier (represented by a dashed arrow). Note that both these processes will result in the actual flux of nitrate from outside to inside, but they give currents of opposite sign.

- **Mechanism of Nitrate Transport Across Membranes**
  - Nitrate transport across biological membranes involves the coupling of charge and solute transport.
  - The translocation of nitrate from high to low concentration is driven by a chemical gradient.
  - The nitrate-binding site is a charged form of the carrier protein, which is then translocated across the membrane.
  - The process is voltage-dependent, increasing at more negative membrane voltages.
  - Nitrate slippage increases at more negative membrane voltages due to the proton-coupled charge translocation.

- **Potential Mechanisms for Nitrate Slippage**
  - Depending on the membrane potential, nitrate transport can be driven by a chemical or electrochemical gradient.
  - At more negative membrane potentials, nitrate transport is driven by an electrochemical gradient.
  - Nitrate slippage is reversible and can occur in both directions.

**Potentia**
nitrate. To explain the apparent differences in $K_{\text{NO}_3}^H$ and $K_{\text{NO}_3}^\text{NM}$ would require 15% nitrate contamination in the nitrite solutions, a figure that is larger than we could measure. This result shows that, like one of the nitrate transport systems in *Chlamydomonas* (13), CRNA can also transport nitrite. Although a specific transporter for nitrite has not been demonstrated in *E. nidulans*, the identification of mutations resulting in hypersensitivity to nitrite implies that it may exist (8, 25, 26). The original *crrA* mutation was selected by growth on chloride-containing medium at mM concentrations (7), but results reported here show that the selection was actually for ClO$_2^-$ resistance (Fig. 2B). The presence of ClO$_2^-$ contaminating chlorite in the original chlorate solutions shows that, like one of the nitrate transport systems in *Arabidopsis* (16, 29, 30), in this work and for AtSUC1 the *crrA* mutation was selected by growth on chloride-containing medium at mM concentrations (7), but results reported here show that the selection was actually for ClO$_2^-$ resistance (Fig. 2B). The presence of ClO$_2^-$ contaminating chlorite in the original chlorate solutions leading to isolation of *crrA*.

The activity of CRNA in oocytes was strongly dependent on the membrane potential, both in providing a direct energy source for transport (with current increasing, as membrane voltage became more negative) but also in changing some of the kinetic properties of the carrier. It was found that $K_{\text{H}}^H$ but not $K_{\text{H}}^\text{NM}$ was voltage-dependent. One of the main features previously described for *in vivo* high affinity nitrate transport, both in *Neurospera* and in plants (6, 27), was the voltage-dependence of the transport system. To demonstrate this voltage-dependence *in vivo*, it was necessary to clamp the membrane voltage at values more negative than −200 mV. However this was not possible in the oocyte experiments because at values more negative than −180 mV there was activation of an endogenous chloride channel in the oocyte plasma membrane (28), which then dominated the I-V curve making it difficult to determine the carrier current. Nonetheless, it has been possible to demonstrate the voltage sensitivity of plant carriers expressed in *Xenopus* oocytes (16, 29, 30). In this work and for AtSUC1 the *Arabidopsis* sucrose transporter (30) there was a similar 1.8-fold increase in the current as membrane voltage was increased from −50 to −150 mV. In contrast, there was almost no voltage-sensitivity of the nitrate cotransport in this range of membrane voltage for *Neurospera*; only at voltages more negative than −150 mV did the voltage-sensitivity become obvious (6). In *Arabidopsis* root hair cells over the same range of membrane voltages (−50 to −150 mV) the nitrate-elicited current increased by 1.8-fold; only at more negative voltages did the effect of membrane voltage become even larger (27). These measurements show that the membrane potential has an important role in regulating CRNA activity, and so any environmental changes that influence this parameter will have effects on nitrate transport.

This model for the reaction cycle of CRNA (Fig. 6) must be very sensitive to the concentration gradient of anion substrate (nitrate) across the plasma membrane. In the oocyte experiments, the cells were previously incubated in a solution containing no nitrate, and the exposure times to nitrate were deliberately minimized. Increasing cytoplasmic concentrations of nitrate can test the model, because the predicted response should be a decrease in the substrate inhibition. There is indirect evidence for this model because the point at the which the onset of the substrate inhibition occurs varies slightly from oocyte to oocyte (compare Figs. 2A, 4, and 5, A–B). This would be consistent with differing internal concentrations of nitrate in each of these oocytes. To test this model, the pattern of anion-substrate inhibition of CRNA transporter activity should be compared for an oocyte that was preincubated in zero nitrate solution and then transferred to nitrate-containing saline. This type of experiment is technically difficult to perform because associated with the nitrate accumulation in the oocyte, there is an acidification of the cytosol that complicates the interpretation of the experiment.

The kinetic model developed here to describe the substrate inhibition has important physiological consequences for the activity of the high affinity nitrate uptake system. The substrate inhibition of CRNA was assayed as a decrease in the measured current, but the actual flux of anions was not determined. When the cytosolic pool of nitrate is depleted, the concentration gradient alone can drive uptake into the cell. In contrast to plants (31), fungi have small vacuolar stores of nitrate to maintain the cytosolic nitrate pools, so the concentration may be depleted more readily. An evolutionary advantage is provided by a nitrate uptake system that requires less energy input by the cell. For example, when a sudden flush of nitrate supply occurs in the environment, there can be a large increase in the external nitrate concentrations. This transport mechanism proves an energetic advantage to the fungus by uncoupling the proton gradient from nitrate uptake. Nitrate influx is driven by the concentration gradient minimizing the requirement for the more energetically expensive coupling to proton fluxes, and yet the same protein can mediate uptake by cotransport at very low external nitrate concentrations. It remains to be seen if these two transport mechanisms are general features of the NNP family or are only found in the fungal members (type II) that are defined by having a large hydrophilic central loop (18).

In conclusion, we have demonstrated that a single protein can function as a high affinity nitrate transport system although the carrier has two different mechanisms for achieving nitrate membrane translocation into the cell.

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