Determination of the Essentiality of the Eight Cysteine Residues of the NrtA Protein for High-Affinity Nitrate Transport and the Generation of a Functional Cysteine-less Transporter†

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ABSTRACT: All eight cysteine residues, C90, C94, C143, C147, C219, C325, C367, and C431, present in transmembrane domains of the *Aspergillus nidulans* NrtA nitrate transporter protein were altered individually by site-specific mutagenesis. The results indicate that six residues, C90, C147, C219, C325, C367, and C431, are not required for nitrate transport. Although alterations of C94 and C143 are less well tolerated, these residues are not mandatory and their possible role is discussed. A series of constructs, all completely devoid of cysteine residues, was generated to permit future cysteine-scanning mutagenesis. The optimum cysteine-less combination was identified as C90A, C94A, C143A, C147T, C219S, C325S, C367S, and C431S. This mutant combination yielded transformant strains with up to 40% of wild-type nitrate transport activity. Furthermore, the K_m value and the level of protein expression were found to be similar to those of the wild-type. This cysteine-less vector should allow us to investigate in detail potentially interesting NrtA amino acids (e.g. identified from homology comparisons) which may be involved in transport, by altering these singly to cysteine and studying such residues by thiol chemistry.

Although nitrate is a common source of nitrogen for a range of organisms from bacteria to higher plants, there are many agricultural, environmental, and even medical issues connected with its use and availability (reviewed in refs *1*−*3*). Nitrate is transported into cells by high-affinity permeases present in most (or even all) nitrate-utilizing organisms. The NrtA permease belongs to a group of nitrate/nitrite transporters forming the NNP[1] family, one of seventeen families that comprise the major facilitator superfamily (MFS) of transporters (*4*−*6*).

While the structures of several MFS proteins have been determined (*7*−*9*), little information is available on the structure of high-affinity nitrate permeases or indeed the role played by individual amino acids in these proteins.

The importance of sulfhydryl groups in the MFS transporter LacY of *Escherichia coli* was determined originally from biochemical data (*10* and references therein). Later site-directed mutagenesis studies of the eight cysteine residues present in the LacY permease showed that only one residue (C154) is important for transport (*11*−*13* and references therein). As well as investigating the essentiality and involvement of cysteine residues per se, this line of research has resulted in the generation of transport proteins devoid of cysteine residues, while retaining some transport activity. These LacY protein variants were used for cysteine-scanning in vitro mutagenesis, an approach employed widely and successfully to explore structure−function relationships of LacY as well as other polytopic membrane proteins, from organisms as diverse as bacteria and humans (*14*−*20*). Of added value, the study of cysteine residues has permitted the creation of a mutant LacY protein that is more stable and amenable to purification for crystallography than the wild-type protein (*9*).

Using site-specific mutagenesis, we have altered, one by one, all eight natural cysteine residues present in putative transmembrane domains (Tm) of the NrtA high-affinity nitrate transporter protein from the lower eukaryote *Aspergillus nidulans* (Figure 1). These eight cysteine residues are C90 and C94, both of which locate within Tm 2, C143 and C147 (Tm 4), C219 (Tm 6), C325 (Tm 7), C367 (Tm 8), and C431 (Tm 10). Our objectives were to (i) determine the essentiality of each of the cysteine residues and (ii) identify strains harboring a functional NrtA protein devoid of all eight cysteine residues for cysteine scanning studies.

MATERIALS AND METHODS

Escherichia coli Strains, Plasmids, and Media. Standard procedures were used for propagation of plasmids, as well

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[1] Abbreviations: Tm, transmembrane domain; NNP, nitrate/nitrite transporter; MFS, major facilitator superfamily.

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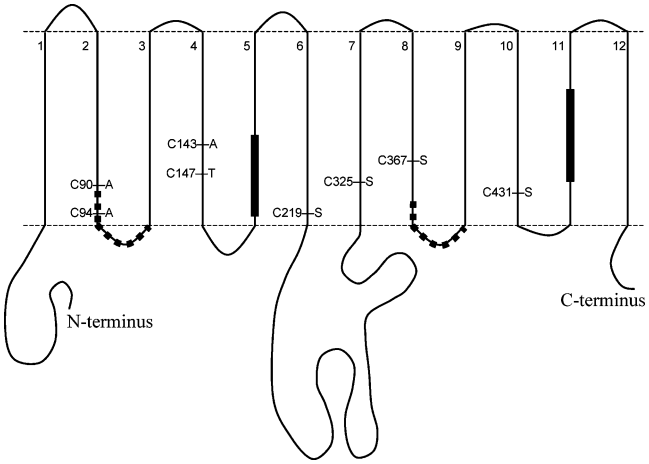

FIGURE 1: Simplified secondary structure model of the high-affinity nitrate transporter NrtA. Transmembrane domains are represented by numbered vertical lines connected by hydrophilic loops. The thick dashed lines in loops 2/3 and 8/9 show the position of the MFS motifs and the black bars in Tms 5 and 11 the positions of the nitrate signatures characteristic of the NNP family. The approximate positions of the eight cysteine residues are indicated with the residue position number to the left of the vertical lines. To the right of each of these is shown the alteration present in the final cysteine-less construct.

as for subcloning and maintenance of plasmids within *E. coli* strain DH5α.

A. nidulans Media. Standard *Aspergillus* growth media and handling techniques were as described before (*21*). Shake flask cultures for nitrate uptake assays were grown in liquid minimal medium (*22*, and as modified by ref *23*).

A. nidulans Strains. Two strains were used as recipients for genetic transformation procedures (see below). One host was the double mutant *nrtA747 nrtB110* (strain JK900), which contains loss-of-function mutations in both nitrate transporter genes, *nrtA* and *nrtB* (*23*). In addition, a triple mutant strain (JK1060) was generated to harbor *nrtA747 nrtB110* mutations as well as the arginine auxotrophic marker *argB2*.

Genetic Transformation Selection Strategy. Two approaches to the selection of transformants were adopted. Initially, direct selection of nitrate-utilizing transformants of strain JK900 was pursued. This allowed us rapidly to assess if the replacement residue was tolerated at the routinely used concentration of 10 mM nitrate. Such a strategy optimized the opportunity for strains with cysteine replacement residues, resulting in very low levels of transport, to display a growth phenotype on nitrate. Although this procedure provides a relatively rapid result, the copy number varies considerably and consequently makes comparative biochemical analyses less meaningful. Therefore, for constructs that showed phenotypic complementation on nitrate, an indirect transformation approach was pursued using strain JK1060. This latter method allows for the independent selection of transformants on the basis of arginine prototrophy, followed by screening such transformants for nitrate utilization (*23*). Moreover, this indirect transformation procedure tends to yield single or at least lower copy integration *nrtA* constructs at the *argB* locus. Single copy transformants at *argB* may be used for comparing the effect of different residue changes.

Genetic Transformation Procedure. The *A. nidulans* transformation procedure was carried out as described before (reviewed in ref *24* and references therein). Conidiospores were grown at 26 °C overnight with orbital shaking at 250 rpm in liquid minimal medium containing 5 mM urea as nitrogen source (strain JK900) and supplemented with 10 mM arginine (strain JK1060). Selection of transformed strains was on osmotic minimal medium containing 10 mM nitrate (JK900), or 5 mM ammonium tartrate (JK1060) at 37 °C for 3 to 7 days. Genetic transformation of the double mutant strain *nrtA747 nrtB110* (strain JK900) was achieved by direct selection on the basis of growth on nitrate as the sole source of nitrogen. Typically, several hundred nitrate-utilizing transformant colonies were obtained using 5 μg of plasmid DNA per transformation. Experiments were repeated to verify the results. If substantial growth was observed on nitrate, an indirect transformation approach was carried out.

Indirect transformation entails the selection for arginine prototrophy in the triple mutant *nrtA747 nrtB110 argB2* (strain JK1060) followed by screening for utilization of 10 mM nitrate to obtain single copy *nrtA* transformants. Copy number and location of integrated constructs was determined in transformants of strain JK1060 by Southern analysis of *Bam*HI-digested genomic DNA (constructs have a single *Bam*HI site) using the *argB* gene as the probe. Integration of the constructs (9.3 kb) into the *argB* locus disrupts the resident approximately 9 kb *Bam*HI fragment to give two fragments of around 11.7 kb and 6.6 kb in the case of a single copy integration. Multiple integrations at *argB* result in hybridization to an additional 9.3 kb fragment (the unit length of the construct) the intensity of which relative to the 11.7 kb and 6.6 kb fragments is indicative of the number of tandemly integrated copies. Single copy strains (derived from JK1060) were used for growth and transport activity analyses.

Molecular Analyses. DNA was isolated using a Nucleon BACC2 Kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.) and RNA using an RNeasy Plant Mini Kit (Qiagen, Crawley, U.K.). The conditions used during Southern analysis were as described previously (*25*). Nucleotide sequencing was determined by automated sequence analysis as described before (*25*).

Generation of Amino Acid Replacement Constructs. Initially cysteine residues were replaced individually with serine, thereby following the approach taken by Kaback and colleagues for the study of the LacY permease (*12*, *13*, and references therein). If poorer growth than wild-type growth resulted (vis-à-vis phenotypic complementation) on 10 mM nitrate as the sole source on nitrogen, vector constructs with further residue changes including alanine, asparagine, glycine, methionine, threonine, or valine were introduced. For generation of mutations by PCR overlap extension (*26*), the template was plasmid pNRTAV5 which comprises the *nrtA* coding region to which is fused in frame at the 3′ end, a sequence encoding the V5 epitope (Invitrogen), and the whole flanked by *Eco*RI sites in pUC19. Details of primers used for mutagenesis are available from the authors. Mutant fragments were inserted into pNRTAV5 using unique restriction sites. Following cloning, all DNA fragments generated by PCR amplification were sequenced to verify the existence of the desired mutation and the absence of other PCR-induced mutations. To produce mutant coding regions with more than one alteration, mutations were combined by ligation of appropriate fragments obtained using unique restriction endonuclease sites.

For expression of the mutant genes in *A. nidulans*, vector pMUT was generated. This plasmid contains 1.3 kb of *nrtA* promoter sequences (including the putative binding sites for regulatory proteins (*27*)), and 350 bp of the *nrtA* terminator, encompassing a unique *Eco*RI site for insertion of mutant coding regions. The vector pMUT also contains as a selectable marker a mutant *argB* gene (i.e. *argB**) to allow targeting of constructs to the *argB* locus, permitting comparison of single copy integrates of *nrtA* (*24*, and references therein). Vector pGPD replaced the 1.3 kb *nrtA* promoter with 1.5 kb of the *A. nidulans gpdA* promoter amplified from pAN8-1 (*28*).

Net Nitrate Transport. This was carried out according to the method of Brownlee and Arst (*29*).

Synthesis of $^{13}NO_3^-$ Tracer. $^{13}NO_3^-$ was generated by proton irradiation of water at the cyclotron facility (Tri-University Meson Facility), University of British Columbia, and contaminating isotopes were removed as described before (*30*, *31*).

Uptake Assays Using the Tracer $^{13}NO_3^-$. Growth of strains and assay of nitrate influx at the routine concentration range of 10 to 250 μM nitrate were as detailed in Unkles et al. (*23*). Briefly, strains were grown for a total of 6.5 h at 37 °C in minimal medium containing 5 mM urea to which was added 10 mM sodium nitrate 100 min prior to harvesting for induction of the nitrate assimilation pathway (*21*−*23*). Following harvesting by filtration and washing to remove nitrate, fungi were resuspended in fresh nitrogen-free minimal medium contained in 250 mL Erlenmeyer flasks. The flasks were shaken in a water bath at 37 °C and inoculated with appropriate volumes of a 10 mM stock of sodium nitrate solution to bring the medium to the desired concentration, and immediately an aliquot of $^{13}NO_3^-$ stock was added (negligible nitrate concentration). After 10 min incubation (transport is linear for at least 30 min), five 10 mL aliquots were filtered individually through glass fiber filters and washed twice with 200 mL of 200 μM nitrate to remove unabsorbed tracer. This procedure has been shown to remove all but negligible traces of $^{13}NO_3^-$ from mycelia and filters. Each filter was introduced into a glass scintillation vial and the accumulated radioactivity determined by gamma counting in a Canberra Packard Gamma Counter. Values for influx are expressed as nmol of nitrate per mg of dry weight (DW) of mycelium per hour (nmol mgDW^{-1} h^{-1}). Each value of K_m or V_{max} was determined by linear regression of a Hoffstee analysis (a plot of *V* against *V*/*S*), with five replicates at each of the seven concentrations used. For each transformant, a completely independent experiment was run twice; results were highly reproducible.

Protein Expression Analysis. For immunological detection, a V5 epitope was fused in frame to the C-terminus of NrtA and plasma membranes were prepared as described (*32*).

RESULTS

Growth and Net Nitrate Uptake Levels in Control Strains. As both single mutant strains *nrtA747* and *nrtB110* mutants (i) grow as wild-type on agar minimal medium with 10 mM nitrate as the sole source of nitrogen (*23*) and (ii) show considerable levels of net nitrate transport for young spore germlings (Table 1), the double mutant *nrtA747 nrtB110* (strain JK900), in which nitrate transport is undetectable, was used as a recipient for constructs generated in this study.

Table 1: Net Nitrate Uptake in Wild-Type, Mutant, and Transformant Strains[a]

strain	net nitrate uptake
wild-type	822 ± 36
nrtA747	210 ± 30
nrtB110	672 ± 69
nrtA747 nrtB110	−7.2 ± 4
MUTwt-T7905	768 ± 18
MUTC94S-T139	96 ± 12
MUTC94A-T209	480 ± 48
MUTC143S-T922	246 ± 24
MUTC143A-T441	282 ± 36
MUTC431S-T301	812 ± 6
GPDwt-T145	920 ± 10
AAAT-T5	306 ± 12

[a] Strains were grown for 6 h in media containing 2.5 mM urea as sole nitrogen source and exposed to 10 mM NO_3^- for 100 min prior to net influx measurements. Wild-type refers to the biotin-requiring auxotrophic mutant (*biA1*). Strains *nrtA747* and *nrtB110* carry single mutations in the *nrtA* and *nrtB* genes, respectively. These mutants were crossed to form the double mutant *nrtA747 nrtB110* (strain JK900). Constructs were transformed into the triple mutant *nrtA747 nrtB110 argB2* (strain JK1060) and transformants harboring a single copy named according to the construct used followed by a transformant number. Further details of constructs and strains are discussed in the text. Values are expressed as nmol mgDW^{-1} h^{-1}.

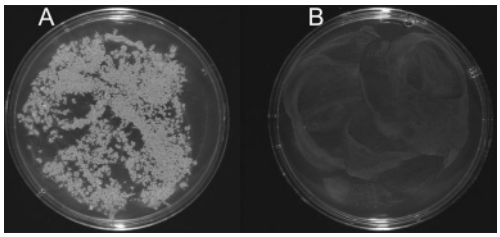

FIGURE 2: Direct selection of transformants. (A) Phenotypic repair of mutant strain JK900 (a double mutant in the two *A. nidulans* transporter genes *nrtA747 nrtB110*) on minimal medium with 10 mM nitrate as the sole nitrogen source after 3 day incubation at 37 °C, with vector pMUTwt containing the wild-type *nrtA* gene. This wild-type level of growth is scored as ++++ in Table 2. (B) Complete lack of growth (similar to untransformed JK900) is observed when transformed with a construct that failed to phenotypically rescue of *nrtA747 nrtB110* double mutant, here shown for example a construct with a Cys94 to Asn change, scored as minus in Table 2).

A wild-type NrtA construct, pMUTwt, was used as the positive control for gene replacement experiments by direct transformation selection into the double mutant *nrtA747 nrtB110* strain JK900 (the selection strategy used is described in detail in Experimental Procedures). Vector pMUTwt yielded vigorously growing colonies (Figure 2A, and growth scored as ++++ in Table 2) following direct selection on nitrate. In contrast, certain NrtA mutant constructs did not yield any growth after transformation (Figure 2B) and consequently were scored as minus (Table 2).

It was also important to determine if strains with the wild-type *nrtA* allele positioned as a single copy at the *argB* locus grew normally and showed wild-type levels of nitrate transport activity. Using the indirect transformation route, vector pMUTwt was transformed into the triple mutant *nrtA747 nrtB110 argB2*, strain JK1060 (see Materials and Methods). Twenty-nine arginine prototrophic transformants were screened for the ability to utilize nitrate as the sole source of nitrogen, similar to a natural wild-type (i.e. a non-recombinant strain with the *nrtA* at its natural site), and of

Table 2: Qualitative Assessment of the Ability of Individual Cysteine Replacement Constructs To Complement Strain JK900 on 10 mM Nitrate[a]

construct	level of growth	construct	level of growth
pMUTwt	++++	pMUTC143S	+
pMUTC90S	+++	pMUTC143A	++
pMUTC90A	++++	pMUTC143N	++
pMUTC90V	+++	pMUTC143T	−
pMUTC94S	+	pMUTC143V	+
pMUTC94A	++	pMUTC147S	++
pMUTC94N	−	pMUTC147A	+
pMUTC94G	+	pMUTC147M	++
pMUTC94V	+	pMUTC147T	+++

[a] Growth scores on minimal selection medium pH 6.5 of transformants with the above constructs are defined approximately as no growth −, poor growth +, intermediate growth ++, good growth +++, and wild-type ++++ growth on agar minimal medium with 10 mM nitrate as the sole nitrogen source. Vector pMUTwt refers to the fact that the *nrtA* gene contains cysteine at the eight natural residue positions, i.e., 90, 94, 143, 147, 219, 325, 367, and 431. Growth level of pMUTwt transformants is used as the positive control.

these, 14 were able to grow as wild-type on nitrate. The DNA of eight transformants was Southern blotted to determine copy number. Four transformants (MUTwt-T7905, MUTwt-T7908, MUTwt-T7920, and MUTwt-T7928) were found to be the result of single copy integration events at the *argB* locus, while the four others were clearly multiple copy. Three single copy pMUTwt transformants, MUTwt-T7905 (Table 1), MUTwt-T7908, and MUTwt-T7928, were assayed for net nitrate transport. These net transport values (around 720−840 nmol $mgDW^{-1}$ h^{-1}) for young spore germlings were similar to the values for strain *nrtB110* (i.e. with *nrtA* wild-type gene at its natural site) (Table 1).

Single Cysteine Residue Replacements. Initially, the effect on growth of alteration of each cysteine residue individually to serine was assessed by the ability of the double mutant transformed with the cysteine replacement constructs to grow on nitrate as sole nitrogen source following direct selection (Table 2). No reduction in growth compared to wild-type was observed when C219, C325, C367, or C431 was replaced by cysteine. Construct pMUTC90S yielded transformants showing good growth albeit somewhat poorer than wild-type, while growth of transformants harboring pMUTC147S appeared intermediate, or poor with pMUTC94S and pMUTC143S. Other alterations of residues C90, C94, C143, and C147 were therefore tested in attempts to improve the growth of resulting transformants. Of these changes shown in Table 2, wild-type growth was observed only for the C90 to alanine substitution while threonine at position 147 allowed good growth. Other changes assessed, including all those to C94 and C143, gave only intermediate, poor, or no growth.

Construction of a Functional NrtA Transporter Devoid of Cysteine Residues. Despite the poor (+, vis-à-vis C94) or intermediate (++, C143 or C147) or even good (+++, C90) growth by single cysteine to serine alterations, respectively, we made a construct (pMUTC1-8S) in which all eight cysteines were changed to serine. However, on direct selection pMUTC1-8S-transformed strains failed to grow (−) on nitrate. This construct as well as constructs with single cysteine replacements (discussed above) was under the control of the natural but weak promoter (*nrtA*) in pMUT (*23*, *33*). Further experimentation utilized vector pGPD containing the strong *gpdA* promoter required for glucose-6-phosphate dehydrogenase expression (*28*) in an attempt to obtain growth of cysteine-less, serine-substituted NrtA strains. However, pGPDC1-8S, in which *nrtA* expression was now governed by the *gpdA* promoter, likewise failed to permit growth.

Table 3: Qualitative Assessment of the Ability of Multiple Cysteine Replacement Constructs To Complement Strain JK900 on 10 mM Nitrate[a]

alteration				
C90	C94	C143	C147	level of growth
S	S	S	S	−
A	A	A	T	++/+++
A	V	A	T	++
A	A	A	T	++
S	A	A	T	+

[a] Growth scores are described in Table 2. The four altered N terminal cysteine residues are given above. Serine replaced the remaining cysteine residues at positions 219, 325, 367, and 431.

Consequently, since the lack of function of cysteine-less, serine-substituted NrtA may have been due to an additive effect, particularly of alterations C90S, C94S, C143S, and C147S, further vector constructs devoid of cysteine were generated based on the growth responses of strains harboring individual residue changes at these positions. All such mutant NrtA were constructed using vector pGPD since the *gpdA* promoter enhanced the net nitrate uptake activity. In this regard, single copy strains harboring pGPDwt (the *gpdA*-controlled wild-type *nrtA* gene) located as before at *argB* were found to have net nitrate uptake values consistently at least 20% higher than those of strains containing pMUTwt (Table 1). While serine was retained at positions 90, 219, 325, 367, and 431 throughout this series of experiments, C94, C143, and C147 were altered to various other residues to yield NrtA constructs with different permutations. However, of these, only the construct with C94A, C143A, and C147T (in the other positions, serine) yielded strains capable of growth on nitrate. Finally, a series of constructs was made in which C90 was altered to alanine and various permutations of C94, C143, and C147 were included (serine at positions 219, 325, 367, and 431). Encouragingly, such constructs as pGPDAVAT and pGPDAANT showed intermediate growth (++), while pGPDAAAT (C90A, C94A, C143A, and C147T with residues 219, 325, 367, 431 remaining as serine) was observed to be best in terms of growth response (+++) of all the constructs generated in direct selection experiments (Table 3).

Strains harboring a single copy of pGPDAAAT located at *argB* were obtained by indirect transformation selection (e.g. transformant designated AAAT-T5), and such mutants confirmed the growth properties of transformants from direct selection (Figure 3). In addition, a multiple copy integration at the *argB* locus (AAAT-T21) was observed (Figure 3). Transformant AAAT-T21 grew better than the single copy strain and was studied in parallel with AAAT-T5. In net nitrate uptake assays, the single copy NrtA cysteine-less strain AAAT-T5 had a value that was approximately 40% of that found for transformant strain MUTwt-T7905 and around 33% of that for transformant GPDwt-T145 expressing the wild-type protein but, as with strain AAAT-T5, under the control of the *gpdA* promoter (Table 1).

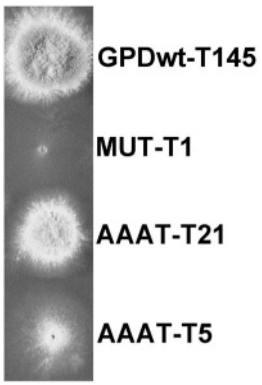

FIGURE 3: Growth tests of transformants of determined copy number integrated at the *argB* locus. Arginine prototrophic transformants of JK1060 grown on minimal medium containing 10 mM nitrate as the sole nitrogen source. GPDwt-T145 is a transformant with a single *nrtA* copy located at the *argB* locus and acts as the positive control for wild-type growth. Strain MUT-T1 has been transformed by the vector pMUT which contains the *argB* gene only, and is therefore the negative control. Transformant AAAT-T5 is a single copy integration of the gene encoding cysteine-less NrtA (i.e. Cys90Ala, Cys94Ala, Cys143Ala, Cys147Thr, Cys219Ser, Cys325Ser, Cys367Ser, and Cys431Ser). Strain AAAT-T21 is a transformant with 5−6 copies of this mutant gene.

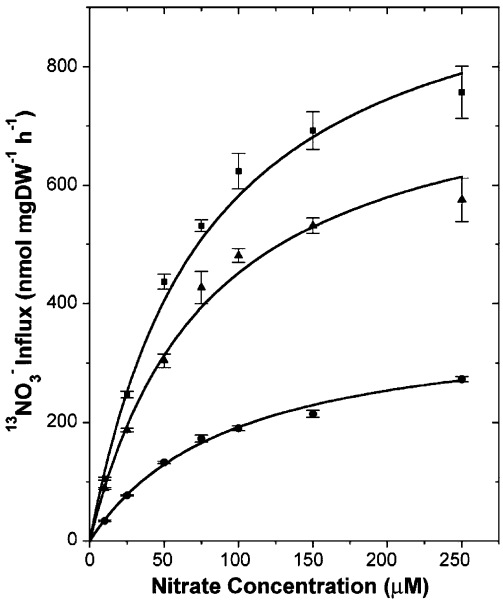

FIGURE 4: $^{13}NO_3^-$ influx kinetic values. Initial rates of $^{13}NO_3^-$ influx for cysteine-less single copy NrtA strain AAAT-T5 (●), cysteine-less multi-copy NrtA strain AAAT-T21 (▲), and single copy wild-type NrtA strain GPDwt-T145 (■) all under *gpdA* expression were measured at lower concentrations up to 250 μM. Further strain details are given in the caption to Figure 3 and in the text.

Kinetics of Nitrate Uptake in Replacement Strains Using Tracer $^{13}NO_3^-$. Initial rates of nitrate influx were measured using the short-lived isotope ^{13}N ($t_{0.5}$ = 10 min) at a NO_3^- concentration range up to 250 μM. The K_m value for the wild-type NrtA protein in the transformant GPDwt-T145 was found to be 77.6 μM while the V_{max} value was 1034 nmol mgDW^{-1} h^{-1} in young mycelium (Figure 4, summarized in Table 4). The cysteine-less mutant protein (i.e. C90A, C94A, C143A, C147T, C219S, C325S, C367S, and C431S) present as one copy in AAAT-T5 showed no significant difference in K_m although V_{max} had markedly decreased compared with the wild-type value. Transformant AAAT-T21 with 5−6 copies of this cysteine-less construct showed a similar K_m value but with the V_{max} value approaching that of the wild-type.

Table 4: Kinetic Constants for $^{13}NO_3^-$ Influx by NrtA Strains[a]

transformant	K_m	V_{max}
GPDwt-T145	77.6 ± 8.6	1034 ± 61
AAAT-T5	94.3 ± 3.5	373.6 ± 8.4
AAAT-T21	79.1 ± 8.8	809 ± 52

[a] Strains were grown for 6 h in media containing 2.5 mM urea as sole nitrogen source and exposed to 10 mM NO_3^- for 100 min prior to influx measurements. K_m (μM) and V_{max} (nmol mgDW^{-1} h^{-1}) values were determined by computer, using direct fits of data to rectangular hyperbolae. AAAT-T5 and AAAT-T21 harbor cysteine-less mutant construct(s), pAAAT (C90A, C94A, C143A, C147T, C219S, C325S, C367S, C431S), while GPDwt-T145 harbors the wild-type construct. The transcriptional expression of *nrtA* in all three transformants is governed by the *gpdA* promoter. Transformant AAAT-T5 has a single *nrtA* gene copy integration, AAAT-T21 has 5−6 copies, and GPDwt-T145 has a single *nrtA* gene copy integration all at the *argB* locus, as determined by Southern blot (unpublished data).

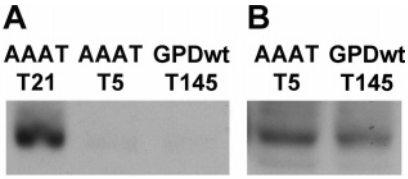

FIGURE 5: Expression of NrtA. Around 50 μg of protein from membrane fractions of strains carrying V5-tagged constructs was subjected to SDS/10% PAGE followed by electroblotting on to Hybond-P membrane (Amersham Biosciences) and probing with HRP-conjugated anti-V5 antibody (Invitrogen) using an ECL Plus Western blotting detection system (Amersham Biosciences). (A) Transformant AAAT-T21 contains multiple copies and transformant AAAT-T5 a single copy of the cysteine-less NrtA construct while GPDwt-T145 refers to a transformant containing the otherwise wild-type NrtA construct. Details of constructs are given in the caption to Figure 3 and in the text. (B) A 10-fold longer exposure of lanes AAAT-T5 and GPDwt-T145 from panel A.

Expression of Mutant NrtA Protein. The level of protein expression in representative transformants containing wild-type or cysteine-less constructs is shown in Figure 5. Protein levels similar to transformant GPDwt-T145 containing one copy of a construct encoding the wild-type NrtA protein were observed in the single copy transformant AAAT-T5. Therefore, the alteration of cysteine residues did not result in changes of NrtA expression. By comparison, NrtA in the multicopy transformant AAAT-T21 was considerably overexpressed.

DISCUSSION

The eight cysteinyl residues in the NrtA permease for high-affinity nitrate transport activity were changed by site-directed mutagenesis (i) individually to determine their essentiality and (ii) in combination to generate a functional NrtA protein completely devoid of cysteine, for the purpose of carrying out future cysteine-scanning mutagenesis experimentation.

Serine replacement of cysteine might be expected to result in minimal disturbance to protein conformation. This indeed appeared to be the case, as judged by growth responses, for the four C-terminal cysteine residues, C219, C325, C367, and C431. None of these residues is conserved in other putative eukaryotic high-affinity nitrate transporters support-

ing the conclusion that they are unlikely to be required for activity of the transporter. Clearly for position 90 also, the sulfhydryl group is similarly unnecessary since a small hydrophobic residue such as alanine can substitute adequately. Considering the lack of conservation of cysteine at this residue position in other putative high-affinity nitrate transporters, this result might be expected.

Mutants with a C147S alteration exhibited only intermediate growth. Like C90, this residue is not well conserved being present at an equivalent position in only five of the 31 eukaryotic proteins surveyed. Within the fungi and algae, threonine is observed most frequently at this position and in the higher plants, methionine. Therefore, C147T and C147M changes as well as a C147A change were assessed. At this position, threonine was accepted affording good growth, better than serine or methionine, but, unlike the C90A alteration, C147A allowed only poor growth of mutants. Therefore it appears that at position 147 a polar amino acid is not mandatory but possibly there is a constraint of side chain bulk, alanine being too small and methionine too large.

As might have been predicted from the high degree of conservation of both C94 and C143 in eukaryotes (29/31 for both), these residues proved the most exacting in terms of substitutions tolerated and none of the substitutions tested permitted wild-type growth. C94 lies within a motif recognized in all MFS proteins although cysteine at the fourth position within the motif is conserved only in members of the NNP family. Its role therefore is unlikely to be integral to the general MFS functioning of this motif, thought to be that of defining conformational changes during transport (*34*). Indeed, in LacY and the tetracycline transporter substitutions of the equivalent residue at this position are well tolerated (*13*, *35*). However, in NrtA, most alterations of C94 resulted in strains unable to grow or growing poorly on nitrate and only change to alanine allowed intermediate growth. Surprisingly, since it is one of the exceptions to conservation of C143, threonine was not tolerated at that position although the other exception, asparagine, with a very similar side chain bulk was accepted to the extent of allowing intermediate growth of mutant strains. The fact that alanine was also accepted to a similar level however indicates that a polar residue is not required at position 143. The results therefore demonstrate that none of the eight cysteinyl residues is essential for activity as each of the eight cysteines may be changed, to at least one other residue, without the complete loss of activity.

Although individual cysteine to serine changes yielded mutants which could grow on nitrate, combination of these alterations in a cysteine-less mutant NrtA did not permit growth even if the mutant gene was under the control of a strong promoter. However, a combination which included the optimum change for each cysteine (in terms of that which allowed maximum growth on nitrate) provided a cysteine-less NrtA with around 40% of wild-type activity. The cysteine-less LacY provides around 30−50% of the wild-type LacY activity (*36*), and so by comparison the AAAT-T5 strain provides a moderately high, measurable baseline activity for future cysteine-scanning mutagenesis. Furthermore, cysteine-less mutants such as AAAT-T5 showed a wild-type K_m value for nitrate. It would appear therefore, since there is no change in the K_m for nitrate, that none of the eight cysteines is involved directly in substrate transport. Neither do any of the cysteines seem to be involved in insertion of the protein in the membrane since protein expression levels of NrtA from the cysteine-less construct are equivalent to those from the wild-type construct, although it is possible to increase protein level and activity in a multicopy strain such as AAAT-T21. The effects on the rate of nitrate uptake suggest instead that cysteines may be involved in conformational changes required for activity of the protein. The most likely candidates in this regard would be well conserved residues C94 (located in Tm 2) and C143 (Tm 4). Residues C94 and C143 may form transient inter-Tm hydrogen bonding stabilizing the protein during nitrate transport although participation of C94 and C143 in inter-Tm interaction would be an advantage but not a prerequisite to NrtA function. Investigation of such possibilities will be the subject of future cysteine-scanning studies.

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