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Nitrate Uptake in *Aspergillus nidulans* and Involvement of the Third Gene of the Nitrate Assimilation Gene Cluster

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In *Aspergillus nidulans*, chlorate strongly inhibited net nitrate uptake, a process separate and distinct from, but dependent upon, the nitrate reductase reaction. Uptake was inhibited by uncouplers, indicating that a proton gradient across the plasma membrane is required. Cyanide, azide, and *N*-ethylmaleimide were also potent inhibitors of uptake, but these compounds also inhibited nitrate reductase. The net uptake kinetics were problematic, presumably due to the presence of more than one uptake system and the dependence on nitrate reduction, but an apparent K_m of 200 μM was estimated. In uptake assays, the *crnA1* mutation reduced nitrate uptake severalfold in conidiospores and young mycelia but had no effect in older mycelia. Several growth tests also indicate that *crnA1* reduces nitrate uptake. *crnA* expression was subject to control by the positive-acting regulatory gene *areA*, mediating nitrogen metabolite repression, but was not under the control of the positive-acting regulatory gene *nirA*, mediating nitrate induction.

One of the most thoroughly studied metabolic processes in eucaryotic microorganisms is nitrate assimilation in the ascomycete *Aspergillus nidulans* (reviewed by Cove [10]). One aspect of this process, nitrate uptake, has, however, received scant attention apart from a few preliminary experiments reported briefly in a review (20). Tightly clustered but discrete genes specify functionally related products in nitrate assimilation by *A. nidulans*. Three clustered genes occur in the order (27) *crnA niiA niaD* in linkage group VIII. *niaD* and *niiA* are the structural genes for nitrate and nitrite reductases, respectively (10), whereas *crnA* is a heretofore uncharacterized gene in which loss of function confers resistance to the toxic analogs chlorate and bromate without any obvious nutritional impairment (27). The phenotypes of deletion mutations covering this region give no reason to suppose that the cluster contains any additional genes (27). Two positive-acting regulatory genes, both recombining freely with the cluster, control expression of *niaD* and *niiA*: *nirA* mediates induction by nitrate and nitrite, and *nirA*$^-$ mutations (loss of function) lead to non-inducibility of nitrate and nitrite reductases and inability to utilize nitrate or nitrite as a nitrogen source (10). *areA* mediates nitrogen metabolite repression, and *areA*^{r} mutations (loss of function) lead to extremely low levels of many activities involved in nitrogen nutrition, including nitrate and nitrite reductases, and inability to utilize nitrogen sources other than ammonium (2, 10, 26). The requirement for the *areA* product for expression of *niaD* and *niiA* is partially alleviated by *nirA*$^{c/d}$ alleles, containing two separate mutations in *nirA*, resulting in constitutivity and nitrogen metabolite derepression, respectively (26).

D. J. Cove (personal communication) originally proposed that the *crnA* gene product might be involved in nitrate uptake in *A. nidulans*, but early attempts to demonstrate defective nitrate uptake by mycelia of *crnA*$^-$ strains were unsuccessful (A. B. Tomsett, Ph.D. thesis, University of Cambridge, Cambridge, England, 1977). We were encouraged to pursue this possibility by the finding that *crnA*$^-$ strains can be distinguished from *crnA*$^+$ strains in two kinds of growth tests on nitrate-containing solid media (unpublished data). First, although *crnA*$^-$ strains utilize even very limiting levels of nitrate normally, they are hypersensitive to a number of growth inhibitors such as Cs^+ and methylammonium when nitrate serves as the nitrogen source, but not on other nitrogen sources, including nitrite. Such inhibitor hypersensitivity characteristically results from mutations reducing the rate of nitrogen source utilization (15, 22). Second, various mutations in *A. nidulans* result in toxicity of nitrate and nitrite or nitrite alone (10, 22, 26), and in double mutants, *crnA* mutations protect against nitrate, but not nitrite, toxicity.

Here we examine some characteristics of net nitrate uptake in *A. nidulans*, demonstrating that a typical *crnA* mutation impairs nitrate uptake in

conidiospores and young mycelia. We believe this to be the first report of a mutation affecting nitrate uptake in a microorganism, such mutations having been reported previously only in the higher plant *Arabidopsis thaliana* (11).

(A preliminary report of this work was presented to the Genetical Society of Great Britain [A. G. Brownlee and H. N. Arst, Jr., Heredity **48**:321, 1982].)

MATERIALS AND METHODS

Strains. All strains of *A. nidulans* carried markers in standard use which have been described previously (6; A. J. Clutterbuck, Aspergillus Newsl. **15**:58–75, 1981; Table 1).

Genetic techniques and growth tests. Genetic techniques followed procedures in standard use (6). Growth testing of *A. nidulans* by stab inoculation onto solid media at 37°C has been described previously (2). The minimal medium described by Cove (7) was used throughout. It contained 10 g of D-glucose per liter as a carbon source. Unless other concentrations are specified, nitrogen sources were added at 10 mM (ammonium, nitrate, nitrite), 5 mM (L-alanine, L-arginine, L-proline), 720 μM (hypoxanthine), or 590 μM (uric acid). Nitrate and nitrite were added as the sodium salts, ammonium was added as the (+)-tartrate, and L-arginine was added as the hydrochloride. Bromate and chlorate were added as the potassium salts. Cesium and methylammonium were added as the chlorides.

Chemicals. Analytical reagent grade chemicals were used wherever commercially available. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 2,4,-dinitrophenol, cycloheximide, valinomycin, and NADPH were obtained from Sigma Chemical Co., Ltd., London. Miracloth was purchased from Calbiochem-Behring Corp., San Diego, Calif.

Conidiospores and mycelia. Conidiospores were harvested from petri dishes containing complete medium (7) into sterile distilled water containing 0.01% Tween 80 and were used either directly for uptake studies or for inoculation for growth of mycelia (at approximately one-fifth plate per flask) in 1-liter polypropylene Erlenmeyer flasks containing 200 ml of liquid medium. The standard nitrogen-free liquid minimal medium of Cove (7) was used throughout. Unless otherwise indicated, it contained 10 g of D-glucose per liter as carbon source. Growth requirements were supplemented at standard levels (7). Nitrogen sources were present as indicated. Urea was used at a final concentration of 5 mM. Mycelia were grown at 37°C in a Gallenkamp Orbital Shaker (Gallenkamp and Co., Ltd., London) with a shaker speed of 200 rpm.

Net nitrate uptake. Nitrate uptake by conidiospores and mycelia was estimated by following the disappearance of nitrate from the medium, a procedure which was successfully employed with both *Penicillium chrysogenum* (14) and *Neurospora crassa* (24). Nitrate concentrations were determined from the absorbance at 204 nm in 5% perchloric acid (5), using an SP 1800 spectrophotometer (Pye-Unicam Instruments Ltd., Cambridge, England). Mycelia were harvested on nylon cloth, washed with prewarmed (37°C), nitrogen-free minimal medium (7), and transferred to 50 ml of nitrogen-free minimal medium (pH 6.5) containing D-glucose and supplemented with (per liter) 10 μg of biotin, 4 mg of *p*-aminobenzoic acid, and, where necessary, 5 mg of pyridoxine hydrochloride in 250-ml Erlenmeyer flasks held at 37°C in a shaking water bath. The mycelial density was usually 1 to 2 mg (dry weight) per ml. After the addition of 500 μM sodium nitrate, nitrate uptake rates were estimated from the decrease in absorbance at 204 nm of acidified samples of media (suitably diluted). Rates were based upon at least four and usually five rapidly filtered 3-ml samples taken within 20 min. Controls from which nitrate was omitted were periodically tested. Any possible contribution to the absorbance by nitrite produced from nitrate was determined by using samples with and without 0.2% sulfamic acid (see reference 5). Application of these controls seldom necessitated significant correction. Flasks were shaken at 100 rpm. Uptake rates were linear over a wide range of nitrate concentrations. For conidial uptake studies, this procedure was slightly modified (see the legend to Fig. 1).

CCCP, FCCP, and valinomycin were dissolved in ethanol at 100 times the final concentration, and, in these cases, the controls also contained 1% ethanol. For inhibitor studies, an additional control was employed which measured absorbance in the presence of inhibitor and the absence of nitrate (in the event that an inhibitor absorbed or caused efflux of a substance[s] absorbing at 204 nm).

Nitrate reductase (NADPH:nitrate oxidoreductase) assay. Harvested mycelia were ground in a mortar on ice with an equal weight of acid-washed sand in 10 volumes of cold extraction buffer, half of which was added midway through grinding. The extraction buffer contained (final concentrations) 100 mM phosphate (sodium salts, pH 7.0), 170 mM NaCl, and 1 mM mercaptoethanol. The crude homogenate was centrifuged at 32,000 × *g* for 30 min, and the supernatant fluid was saved. Nitrate reductase (EC 1.6.6.3) was

TABLE 1. Strains used

Relevant genotype	Additional markers[a]	Reference or origin
Wild type	*pabaA1*	2, 6
crnA1	*pabaA1*	27
cnxG2	*biA1*	8, 9, 10, 21
niaD17		9, 10, 21
niiA4	*biA1*	10, 21
nirA101$^{c/d}$	*biA1*[b]	10, 26
nirA101$^{c/d}$	*pabaA1*[b]	10, 26
nirA101$^{c/d}$ *crnA1*	*pabaA1*	Isolate from cross
nirA103$^{c/d}$	*biA1 fwA1*	26
nirA113$^{c/d}$	*pyroA4*	26

[a] *pabaA1*, *biA1*, and *pyroA4* are requirements for *p*-aminobenzoate, biotin, and pyridoxine, respectively. *fwA1* results in fawn conidial color. None of these markers affects nitrogen metabolism. Growth requirements were fully supplemented as described by Cove (7).

[b] The *biA1* strain was used for conidial uptake studies, and the *pabaA1* strain was used for mycelial uptake studies and enzyme assays. This choice was arbitrary, as the *biA1* and *pabaA1* mutations have no effect on these activities.

assayed by following the oxidation of NADPH at 340 nm spectrophotometrically in a 1-ml assay mixture containing 1 nmol of flavin adenine dinucleotide, 200 nmol of NADPH, 10 μmol of $NaNO_3$, and 50 μmol of (sodium) orthophosphate (pH 7.0) and, typically, 50 μl of cell-free extract. Assays were conducted at 32°C and were initiated by adding the nitrate after measuring (and correcting for) the nitrate-independent oxidation of NADPH by the extract. This correction ranged from approximately 2% for the highest nitrate reductase activities reported here to slightly under 15% for the lowest activities.

Nitrite reductase (NADPH:nitrite oxidoreductase) assay. Crude extracts of freshly harvested mycelia were prepared as described for nitrate reductase, but in a pH 8.6 buffer containing (final concentrations) 50 mM (sodium) pyrophosphate, 33% (vol/vol) glycerol, 10 mM L-cysteine, and 1 mM flavin adenine dinucleotide. Nitrite reductase (EC 1.6.6.4) was assayed by following the oxidation of NADPH at 340 nm spectrophotometrically in a 1-ml assay mixture containing 2 μmol of sodium nitrite, 250 nmol of NADPH, and 100 μmol of (sodium) phosphate (pH 7.0) at 25°C. The reaction was initiated by adding 50 μl of cell-free extract (containing the flavin adenine dinucleotide essential for the reaction).

Protein determination. Soluble protein in extracts was determined (16) by using crystalline bovine serum albumin as standard. Soluble protein accounts for approximately 12% of mycelial dry weight (unpublished data). Total protein in conidiospore suspensions (see Fig. 1c) was determined by the same method after solubilization of samples in 100 mM NaOH.

RESULTS

Nitrate and nitrite reductase levels in *crnA1* strains. Under growth conditions resulting initially in maximal induction, the *crnA1* mutation did not lower levels of either enzyme (Table 2). The modest but consistent elevation in enzyme activities shown by the *crnA1* strain in Table 2 might result from reduced self-nitrogen metabolite repression if *crnA1* reduces nitrate uptake, limiting conversion of nitrate to the repressing metabolite.

Nitrate uptake by conidiospores. Freshly harvested conidiospores of *A. nidulans* wild type (whether or not suspended overnight in dilute Tween 80 to enhance their wettability) exhibited net uptake of nitrate only after a lag of up to 2 h at 37°C (Fig. 1a). Acquisition of net nitrate uptake capacity was prevented by the protein synthesis inhibitor cycloheximide and did not occur in conidiospores of a *niaD17* strain lacking nitrate reductase (Fig. 1a). Metabolism of nitrate beyond nitrite is not, however, required; conidiospores of a *niiA4* strain lacking nitrite reductase exhibited nearly normal acquisition of nitrate uptake capacity (data not shown). Appearance of conidial net nitrate uptake capacity required an exogenous carbon and energy source (Fig. 1b). Comparison of Fig. 1a with 1b shows that preincubation of conidiospores in glucose solution allowed the appearance of uptake capacity to commence immediately upon addition of nitrate. This indicates that the lag is distinct from nitrate induction. After the lag period, net nitrate uptake capacity appeared sooner in a *nirA101*$^{c/d}$ strain than in the wild type (Fig. 1c). Addition of an equimolar concentration of ammonium reduced net uptake capacity in the wild type to ca. 5% of the level found on addition of nitrate alone (Fig. 1c). In identical conditions, an *nirA101*$^{c/d}$ strain had ca. 40% as much net uptake capacity when NH_4NO_3 replaced $NaNO_3$ (data not shown).

TABLE 2. Nitrate-assimilating enzymes in the wild type and a *crnA1* strain[a]

Strain	Nitrate reductase activity[b]		Nitrite reductase activity[b]	
	8 h	17 h	8 h	17 h
Wild type	270 ± 71	56 ± 8	203 ± 14	57 ± 3
crnA1	351 ± 67	88 ± 13	274 ± 34	74 ± 2

[a] Mycelia were grown in supplemented glucose minimal medium, with 20 mM nitrate as the nitrogen source, at 37°C for 8 or 17 h, as indicated.

[b] Enzyme activities are given as nanomoles of NADPH oxidized per minute per milligram of protein. Values are mean ± standard deviation (SD) of at least three independent experiments.

crnA1 reduced net nitrate uptake by conidiospores approximately threefold (Table 3). Conidiospores were preincubated to avoid the problem of some variability in length of the lag period, and *nirA101*$^{c/d}$ strains were used to exploit the high, linear initial rate of acquisition of uptake capacity resulting from this allele (Fig. 1c).

Nitrate uptake by mycelia. Net nitrate uptake capacity was inducible by nitrate in wild-type mycelia (Table 4; Fig. 2). Nitrite (at 10 mM) is also a good inducer of net nitrate uptake capacity, but variability in the degree of induction, possibly because of the reactivity and slight toxicity of nitrite, precluded its use. As in conidiospores, functional nitrate reductase was essential for net nitrate uptake activity both in young (grown for 8 h at 37°C) and older (grown for 16 h at 37°C) mycelia, because under inducing growth conditions (Table 4), neither a *niaD17* nor a *cnxG2* strain had any nitrate uptake activity whatsoever. *niaD17* is a mutation in the structural gene for the nitrate reductase apoprotein and leads to chlorate resistance and constitutive syntheses of nitrite reductase and ancillary activities associated with nitrate reductase, whereas *cnxG2* is a mutation in a gene involved in biosynthesis of a molybdenum-containing cofactor common to nitrate reductase

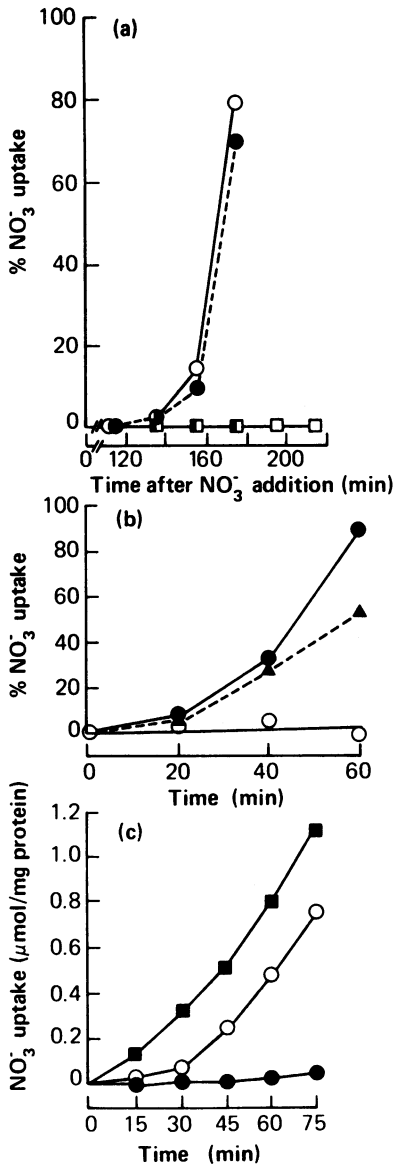

FIG. 1. Net nitrate uptake by conidiospores. (a) Conidiospores were harvested into distilled water containing 0.01% Tween 80, filtered through Miracloth to remove mycelial debris, washed with distilled water using low-speed centrifugation for sedimentation, and suspended in distilled water. Before assay, one sample of wild-type conidiospores was left overnight in 0.01% Tween 80 at 4°C. Approximately 5×10^8 conidiospores were suspended in 50 ml of supplemented glucose minimal medium with or without 10 μg of cycloheximide, and 200 μM nitrate was added. Samples (1.5 ml) were filtered rapidly over a vacuum. Nitrate uptake is expressed as a percentage of the initial concentration (200 μM). Symbols: ○, freshly harvested wild-type conidiospores; ●, wild-type conidiospores left overnight in Tween 80; ■, freshly harvested wild-type conidiospores in the presence of cycloheximide; □, *niaD17* strain. (b) Wild-type conidiospores (1.8×10^7) were suspended in 25 ml of the various nitrogen-free preincubation media shown for 4.5 h before the addition of 200 μM $NaNO_3$ at time zero. For comparison with distilled water, no pH adjustment was made to minimal media in this experiment, so that its pH was approximately the same as that of the distilled water (pH 5.0). Symbols: ○, supplemented minimal medium lacking a carbon source; ●, supplemented minimal medium containing 1% glucose; ▲, distilled water containing 1% glucose. (c) Conidiospores (2.5×10^9) were suspended in 50 ml of supplemented glucose minimal medium and preincubated for 150 min before the addition of 200 μM NO_3^- (as the Na^+ or NH_4^+ salt) at time zero. Symbols: ●, wild type plus NH_4NO_3; ○, wild type plus $NaNO_3$; ■, *nirA101*$^{c/d}$ plus $NaNO_3$.

and two purine hydroxylases and does not lead to chlorate resistance or constitutive syntheses of nitrite reductase and ancillary activities associated with nitrate reductase (8–10, 21). Pateman and Kinghorn (20) have observed a lack of accumulation of $^{15}NO_3^-$ by mycelia of a *niaD17* strain.

In young (7 to 9 h) mycelia, there was a three- to fourfold reduction in net nitrate uptake capacity in a *crnA1* strain as compared with the wild type in inducing growth conditions (Table 4; Fig. 2a). In older mycelia (16 h), there was little or no difference between *crnA1* and wild-type strains (Table 4). Nitrate-induced net nitrate uptake activity varied with mycelial age in an oscillatory fashion (Fig. 2b).

Data in Fig. 2a compare the kinetics of induction of nitrate reductase as well as of net nitrate uptake capacity in wild-type and *crnA1* strains. The kinetics of induction of nitrate reductase in wild type grown on urea as the nitrogen source at 37°C were very similar to those at 25°C described by Cove (8). Induction kinetics of net nitrate uptake capacity in the same mycelia appeared to differ, suggesting nonidentity of nitrate reductase and the nitrate uptake system(s). More convincing evidence for this nonidentity was provided by several experiments in which the wild type was grown on nitrate as the nitrogen source: under these conditions, net nitrate uptake activity was very low (less than 2 nmol/min per mg [dry weight]), whereas nitrate reductase activity was maximally induced. (The diminished nitrate reductase levels and induction lag in the *crnA1* strains seen in Fig. 2a are typical of results obtained in suboptimal induction conditions.)

Further evidence for the nonidentity of nitrate reductase and the nitrate uptake system(s) is contained in Table 5. Three *nirA*$^{c/d}$ alleles, including the most extreme alleles available, *nirA103*$^{c/d}$ and *nirA113*$^{c/d}$ (26), led to significant degrees of nitrogen metabolite derepression of nitrate reductase, with no derepression of the uptake system(s) evident. (The low residual up-

TABLE 3. Nitrate uptake and nitrate reductase activities in *nirA101*$^{c/d}$ strains with and without *crnA1*

Strain	Activity at the following developmental stage[a]:					
	Conidiospores[b]		8-h mycelia[c]		16-h mycelia[c]	
	Nitrate reductase	Nitrate uptake	Nitrate reductase	Nitrate uptake	Nitrate reductase	Nitrate uptake
nirA101$^{c/d}$	ND[d]	5.9 ± 0.4	241 ± 4	16.0 ± 0.2	923 ± 11	23.3 ± 0.6
nirA101$^{c/d}$ *crnA1*	ND	2.0 ± 0.6	243 ± 2	7.0 ± 0.1	826 ± 194	19.6 ± 0.9

[a] Nitrate reductase activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein and is the average of at least two experiments ± SD. Nitrate uptake rates are expressed as nanomoles of NO_3^- removed per minute per milligram (dry weight) and are averages of at least two experiments ± SD.

[b] Freshly harvested conidiospores were preincubated for 4 h in nitrogen-free supplemented glucose minimal medium at 37°C, collected by centrifugation, and suspended in 20 ml of supplemented glucose minimal medium; 500 μM $NaNO_3$ was added. Nitrate uptake was followed as described in the text.

[c] Mycelia were grown in supplemented glucose minimal medium, with urea as the nitrogen source, at 37°C for 8 or 16 h, as indicated. Nitrate uptake rates were determined immediately by using half of the freshly harvested mycelia, and the remainder was frozen at −15°C for subsequent (within a few days) assay of nitrate reductase.

[d] ND, Not determined.

take activities measured in the *nirA101*$^{c/d}$ and *nirA113*$^{c/d}$ strains grown on ammonium probably result from rapid derepression during harvesting of the mycelia and the short incubation in uptake medium.)

Comparison of data in Tables 3, 4, and 5 showed that *nirA*$^{c/d}$ alleles led to a high constitutive level of mycelial net nitrate uptake capacity. This provided an opportunity to investigate the effect of *crnA1* under conditions in which induction was unnecessary. The *nirA101*$^{c/d}$ *crnA1* double mutant had less than half of the net nitrate uptake activity of the *nirA101*$^{c/d}$ *crnA*$^+$ strain in young (8 h) mycelia, but this difference was much less in older (16 h) mycelia (Table 3). Nitrate reductase levels in the two strains were comparable.

Characterization of the mycelial nitrate uptake system(s). Net nitrate uptake was inhibited by various metabolic inhibitors including cyanide, azide, *N*-ethylmaleimide, and the protonophores CCCP, FCCP, and 2,4-dinitrophenol (Table 6). 2,4-Dinitrophenol (500 μM) inhibited nitrate reductase only by approximately 18% in crude extracts, and 100 μM CCCP and 10 μM FCCP had no effect (A. G. Brownlee, unpublished data). That CCCP and FCCP did not affect nitrate reductase activity at 10 times a concentration at which they strongly inhibited nitrate uptake (Table 6) suggests that they act directly on transport.

The effect of pH on net nitrate uptake is shown in Fig. 3. Strains carrying *nirA101*$^{c/d}$ were used to avoid the requirement for induction and to enhance uptake activities. In 8-h mycelia of the *nirA101*$^{c/d}$ *crnA*$^+$ strain, net nitrate uptake was high between pH 4.0 and 7.0 (Fig. 3a). Activity fell rapidly outside this range (although nitrate was ionized throughout the pH range examined). The profile of pH dependence in 8-h mycelia of the *nirA101*$^{c/d}$ *crnA1* double mutant was somewhat different, lower between pH 4.0 and 8.5, but most markedly at pH 4.0 (Fig. 3a). In 17-h (at 37°C) mycelia, the two strains showed almost identical pH dependence (Fig. 3b). Functional nitrate reductase is a prerequisite for net nitrate uptake at any pH; no uptake was observed in a *niaD17* strain at pH 4.2 with the use of 8- or 16-h (at 37°C) mycelia (in addition to the dependence on nitrate reductase under standard assay conditions at pH 6.5 noted above).

Chlorate was a potent and rapid inhibitor of nitrate uptake by 16-h (at 37°C) mycelia in both the wild type and a *crnA1* strain (Fig. 3c). Similar chlorate inhibition was observed in 8-h wild-type mycelia, in which 1 mM chlorate inhibited uptake of 500 μM nitrate by 80%. The extent of chlorate inhibition was apparently pH dependent, however. In a preliminary experiment (data not shown), chlorate was markedly more inhibitory at pH 4.2 than at pH 7.0 in both 8- and 17-h mycelia from a *nirA101*$^{c/d}$ strain.

TABLE 4. Nitrate uptake in the wild type and a *crnA1* strain

Age of mycelia at harvesting (h)[a]	Concn of nitrate added (mM)[b]	Nitrate uptake rate[c] in:	
		Wild type	*crnA1*
7–8	0	1.5 ± 1.2	<1.0
16	0	<1.0	<1.0
7–8	10	11.6 ± 3.0	2.8 ± 0.7
16	10	12.3 ± 3.0	12.4 ± 1.8

[a] Mycelia were grown in supplemented glucose minimal medium, with urea as the nitrogen source, at 37°C.

[b] $NaNO_3$ (10 mM) was added 100 min before harvesting.

[c] Rates are expressed as nanomoles of NO_3^- removed per minute per milligram (dry weight) and are the means ± SD from at least three independent experiments.

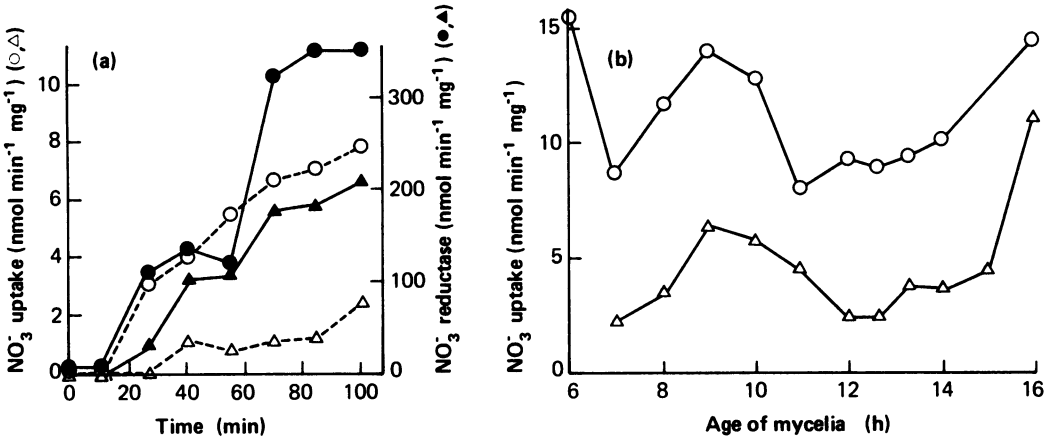

FIG. 2. Induction of mycelial nitrate uptake capacity. (a) Mycelia were grown for 9 h in supplemented glucose minimal medium, with urea as the nitrogen source, at 37°C. At time zero, $NaNO_3$ (5 mM) was added to all flasks. Contents of separate flasks were harvested at each time point, and nitrate uptake activity and nitrate reductase were determined. (b) Mycelia were grown for the times indicated. $NaNO_3$ (10 mM) was added 100 min before harvesting. NO_3^- uptake was determined in the standard assay. Each point was obtained by using a separate flask. Variation between flasks of one strain at any given time was less than 10% (determined separately). Symbols: ○, ●, wild type; △, ▲, *crnA1*; ○, △, nitrate uptake activity; ●, ▲, nitrate reductase activity. In both (a) and (b), uptake rates are expressed per milligram (dry weight), whereas nitrate reductase activities are expressed per milligram of soluble protein.

Chlorate is a weak inhibitor of nitrate reductase (17).

Using the standard uptake assay with 8- and 17-h mycelia of a *nirA101*$^{c/d}$ strain grown with 5 mM urea as the sole nitrogen source, nitrate uptake proceeded at the same rate in the presence or absence of 10 mM ammonium. This experiment was repeated, using 8- and 17-h mycelia grown on 10 mM L-glutamate or 17-h mycelia grown on 10 mM L-phenylalanine as the sole nitrogen source, with the same result. Apparently, therefore, ammonium ion does not act as an uncoupler of nitrate transport.

Analysis of the kinetics of net nitrate uptake proved problematic, presumably as a result of the relative insensitivity of the assay, the dependence of uptake on functional nitrate reductase, and the possible presence of more than one uptake system. Repeated attempts to determine the affinity of the nitrate uptake system in older mycelia have revealed complex kinetics and nonlinear double reciprocal plots. However, the system(s) did exhibit saturation above 1 mM nitrate (Fig. 3d). A Hofstee plot (Fig. 3d insert) indicated an apparent K_m of approximately 200 μM, but the V_{max} value was found to be subject to some variation. No metal cation requirement for uptake was evident, nor was there any apparent effect of low ionic strength. Uptake proceeded equally well in glass-distilled water plus D-glucose and in 20 mM phosphate (as the potassium salts) buffer (pH 6.5) plus glucose plus 0 to 50 mM sodium or lithium chloride as in standard minimal medium.

Regulation of *crnA* expression. Although nitrate uptake was inducible (Table 4; Fig. 1a), it

TABLE 5. Effects of growth in the presence of ammonium on nitrate reductase and nitrate uptake levels in *nirA*$^{c/d}$ strains

Nitrogen source[a]	Activity in strain:					
	nirA101$^{c/d}$		*nirA103*$^{c/d}$		*nirA113*$^{c/d}$	
	Nitrate reductase	Nitrate uptake	Nitrate reductase	Nitrate uptake	Nitrate reductase	Nitrate uptake
5 mM urea	324 ± 2	12.5 ± 5.0	811 ± 82	14.4 ± 8.5	770 ± 158	16.0 ± 0.8
5 mM urea + 20 mM NH_4^+	52 ± 1	<0.1	158 ± 1	<0.1	174 ± 18	<0.1
20 mM NH_4^+	83 ± 36	0.6 ± 0.8	178 ± 18	<0.1	212 ± 61	1.1 ± 1.6

[a] Mycelia were grown for 8 to 9 h at 37°C in supplemented glucose minimal medium with the nitrogen source(s) indicated. Other details were as described for mycelia in Table 3.

TABLE 6. Nitrate uptake by mycelia of a *nirA101*$^{c/d}$ strain in the presence of inhibitors

Addition[a]	Relative nitrate uptake rate (%)[b]	
	8 h[c]	17 h[c]
None	100	100
Valinomycin (10 μg/ml)	ND[d]	82
2,4-Dinitrophenol (500 μM)	26	59
CCCP (10 μM)	6	20
FCCP (1 μM)	ND	26
NaN_3 (1 mM)	<5	<1
KCN (1 mM)	ND	<1
N-Ethylmaleimide (1 mM)	<5	<1

[a] Additions were made to the standard nitrate uptake assay described in the text.

[b] Control rates (no additions) were 8 h, 9.6 ± 1.1; 17 h, 14.1 ± 0.7 nmol of NO_3 per min/mg (dry weight) ± SD. Rates shown in the presence of inhibitors are the averages of duplicate determinations, none of which varied >7% about the mean.

[c] Mycelia of the *nirA101*$^{c/d}$ strain were grown in supplemented glucose minimal medium, with urea as the nitrogen source, for 8 or 17 h at 37°C.

[d] ND, Not determined.

cannot as yet be determined whether synthesis of the *crnA* product itself is inducible or whether this apparent inducibility stems entirely from the inducibility of nitrate reductase. Nitrogen metabolite repression of the syntheses of gene products essential for nitrate uptake, including presumably *crnA*, is, however, distinct from nitrogen metabolite repression of nitrate reductase (Table 5). A comparison of the phenotypes of *crnA1* and *crnA*$^+$ strains carrying in addition various regulatory mutations yielded further information on the regulation of *crnA* expression.

crnA1 was additive in double mutants with *nirA1*$^-$. For example, on glucose minimal medium containing 5 mM L-arginine as the nitrogen source, *crnA1 nirA1*$^-$ double mutants were clearly more resistant to the toxicity of 20 mM chlorate than were single mutants carrying either mutation. As *nirA1*$^-$ is a nonleaky *nirA* mutation (9, 10) and therefore probably results in complete loss of *nirA* product function, the additivity of *crnA1* and *nirA1*$^-$ indicates that expression of *crnA* is largely, possibly completely, independent from *nirA* control. This conclusion is supported by data in Table 5 indicating that, although they relieved nitrogen metabolite repression of nitrate reductase synthesis, *nirA101*$^{c/d}$, *nirA103*$^{c/d}$, and *nirA113*$^{c/d}$ did not lead to nitrogen metabolite derepression of net nitrate uptake capacity.

Double mutants carrying *crnA1* and an *areA*^{r} mutation were constructed by using two different *areA*^{r} alleles. *areA18*^{r} is a complete loss of function allele resulting from a translocation breakpoint (1, 22), whereas *areA1*^{r} is thermosensitive for the utilization of certain nitrogen sources (2). Three independent double mutants of each genotype were definitively identified by their ability to yield *crnA1 areA*$^+$ progeny upon outcrossing to a wild-type strain. These double mutants were indistinguishable from corresponding *areA*^{r} single mutants in a range of growth tests at 37°C, testing nitrogen source utilization and resistance to chlorate and bromate. At 25°C, a permissive temperature, *crnA1 areA1*^{r} strains were readily distinguishable from *areA1*^{r} single mutants by their inability to utilize nitrate, although they retained the ability to utilize nitrite, hypoxanthine, uric acid, and L-proline. This phenotype is consistent with involvement of *crnA* in nitrate but not nitrite uptake. The epistasis of *areA*^{r} mutations to *crnA1* suggests that the *areA* gene product mediates nitrogen metabolite repression of synthesis of the *crnA* product.

The location of *crnA* in a gene cluster with *niaD* and *niiA* (27) is intriguing. One approach to consideration of whether *crnA* is expressed via a polycistronic mRNA is to examine expression of *crnA* in strains carrying *nis-5*, a *niiA* promoter/initiator mutation associated with an insertional translocation (3, 22). In *nis-5* strains, the relevant gene configuration is *crnA niiA* · · · *niaD*, as compared with *crnA niiA niaD* in translocation-free (*nis*$^+$) strains. The proximity of *crnA* to one of the translocation breakpoints increased the difficulty of constructing *crnA1 nis-5* double mutants. This was achieved by selecting *puA*$^+$ *niiA*$^+$ recombinants from a cross of relevant partial genotype *puA2 crnA1* × *niiA203 nis-5* (consult reference 3 for methods of genetic manipulation of *nis-5*). In growth tests, *nis-5* had no effect on chlorate and bromate toxicities, and the differences in phenotype between *crnA1 nis-5* and *crnA*$^+$ *nis-5* strains were the same as those between *crnA1 nis*$^+$ and *crnA*$^+$ *nis*$^+$ strains. Given that *niiA* is almost certainly transcribed towards *crnA* (3), this shows that *crnA* is not to any significant degree expressed via a *niaD niiA crnA* tricistronic messenger.

DISCUSSION

Evidence is presented that, although dependent upon intracellular nitrate reduction, net nitrate uptake is a separate and distinct process. It is sensitive to specific inhibitors, saturable at high concentrations of nitrate, and subject to metabolic and developmental regulation. It has a characteristic pH dependence and is partially defective in strains carrying the *crnA1* mutation. The ability of *crnA1* strains to take up nitrate suggests the existence of more than one mode of nitrate uptake. The broad pH dependency is

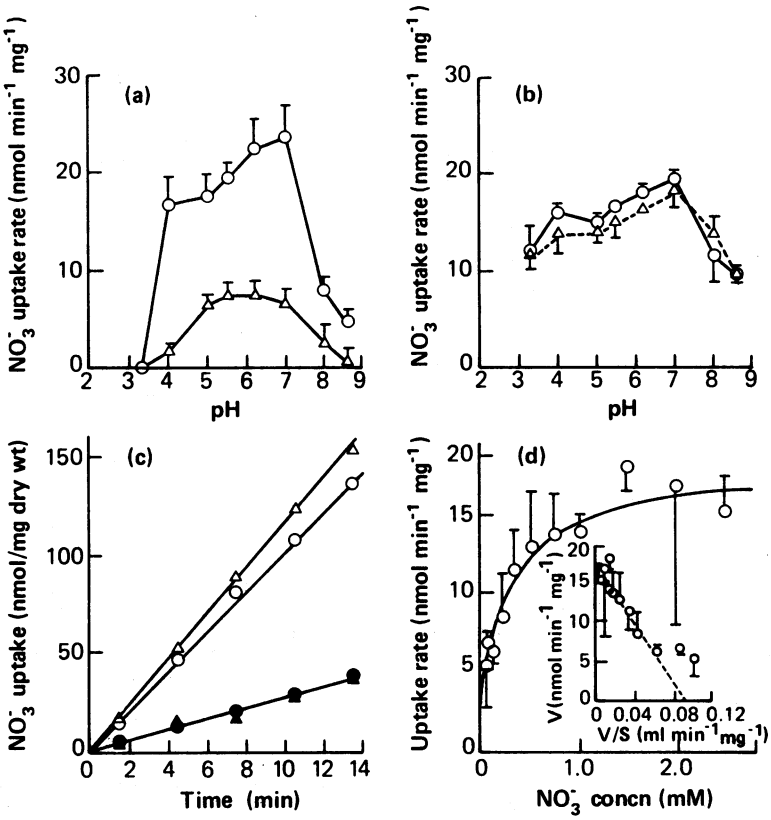

FIG. 3. Properties of the mycelial nitrate uptake system. Mycelia were grown in supplemented glucose minimal medium, with urea as the nitrogen source, at 37°C. pH dependence of nitrate uptake by 8-h (a) or 17-h (b) mycelia was determined by using 500 μM $NaNO_3$ in 20 mM phosphate (as the potassium salts) buffer, at the pH indicated, in the presence of 1% glucose. Values are the means of at least three independent experiments; bars denote 1 SD. Symbols: ○, *nirA101*$^{c/d}$; △, *nirA101*$^{c/d}$ *crnA1*. (c) Mycelia of the wild-type (○, ●) and *crnA1* (△, ▲) strains were grown for 16 h. For the purpose of induction, 10 mM $NaNO_3$ was added 3 h before harvesting. Using the standard assay, uptake of 500 μM nitrate was determined in the presence (●, ▲) and absence (○, △) of 1 mM $KClO_3$. (d) Concentration dependence of nitrate uptake was determined in 17-h mycelia of the *nirA101*$^{c/d}$ strain. The concentrations of $NaNO_3$ indicated were used in the standard uptake assay. Points are the means of three typical experiments. Bars denote 1 SD.

consistent with this notion, and the kinetics of nitrate uptake suggest systems with different affinities for nitrate. Evidence for the existence of two assimilatory nitrate transport systems in *Klebsiella pneumoniae* has been reported (25).

The *crnA1* mutation reduces net nitrate uptake by conidiospores and young mycelia, but not by older mycelia, perhaps explaining why Tomsett (Ph.D. thesis) was unable to detect any effect of *crnA1* on nitrate uptake. Thus, nitrate uptake by *A. nidulans* might bear some resemblance to sulfate uptake by *N. crassa*, in which there are two genetically and biochemically distinct permeases, one operative in conidiospores and another operative in mycelia (18). In addition to this developmental regulation, *crnA* is apparently under the control of the *areA* regulatory gene mediating nitrogen metabolite repression. The available evidence suggests, however, that *crnA* is not under the control of the *nirA* regulatory gene mediating nitrate and nitrite induction. *In vivo* evidence suggests that *crnA* is not expressed via a tricistronic transcript of the nitrate assimilation gene cluster.

The electrogenic proton-translocating ATPase of fungal plasma membranes probably functions in the transport of both ions and uncharged compounds into the cell against a large electric potential or concentration gradient (reviewed in reference 13). The effect of uncouplers on net nitrate uptake in *A. nidulans* observed here might implicate the proton gradient in supplying energy for the transport of the nitrate anion against a probably large membrane potential (which in *N. crassa* can reach more than −300 mV, inside negative [23]). It is also pertinent that

N-ethylmaleimide inhibits the purified *N. crassa* plasma membrane proton-translocating ATPase (4). However, *N*-ethylmaleimide, cyanide, and azide are very potent inhibitors of nitrate reductase, both purified and in crude extracts (12, 17, 19; A. G. Brownlee, unpublished data). Thus, the mode(s) of action of these inhibitors in blocking nitrate transport remains equivocal: a secondary consequence of inhibition of nitrate reductase, a more direct effect on transport, or both. Depolarization studies have provided evidence for nitrate-proton contransport in *Lemna gibba* (28).

The potential nitrate-reducing activity (measured in vitro at saturating nitrate and NADPH concentrations) was in excess, often considerable excess, of net nitrate uptake capacity of the same cells in all strains and growth conditions employed here (e.g., Tables 3 and 5; Fig. 2a). The best available estimate of the K_m for nitrate of purified nitrate reductase from *A. nidulans* is 80 μM (17). This suggests that our estimate of 200 μM for the K_m for nitrate uptake is a measure of the saturability of an uptake system and not of nitrate reductase.

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