

Structure–function analysis of NADPH: nitrate reductase from *Aspergillus nidulans*: analysis of altered pyridine nucleotide specificity *in vivo*

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Nitrate reductase (NaR) catalyses the reduction of nitrate to nitrite via a two-electron transfer. In fungi, the electron donor for NaR is NADPH whereas plants can have two enzymes, NADH:NaR and a bispecific NAD(P)H:NaR. PCR mutagenesis was employed to introduce mutations into the *niaD* gene of *Aspergillus nidulans* in order to identify residues involved in co-enzyme specificity. The *niaD3000* mutation (NiaD T813D, K814Q) altered co-enzyme specificity: the new enzyme had high levels of NADH:NaR activity *in vitro*, whilst all NADPH-associated activity was lost. However, strains carrying this mutation did not grow on nitrate. Enzyme assays suggested that this was not due to inhibition of the mutant enzyme by NADPH. All revertants of the *niaD3000* mutants had restored NADPH activity and lost NADH activity. Sequence analysis of these revertants showed that they all contained a single amino acid change at Asp-813, suggesting that this position is crucial to co-enzyme specificity. Further studies have shown that the mutant enzyme was not protected from deactivation by either co-factor in cell-free extracts (unlike the wild-type), and that induction of the glucose-6-phosphate dehydrogenase occurred independently of NADPH levels. These data highlight the importance of functional tests *in vivo* under physiological conditions.

Keywords: *Aspergillus nidulans*, nitrate reductase, site-directed mutagenesis, NADPH specificity, pentose phosphate pathway

INTRODUCTION

In eukaryotes, nitrate reductase (NaR) catalyses the first step in nitrate assimilation, the conversion of nitrate to nitrite. NaR is a multicentre redox protein that has three distinct regions (Kinghorn & Campbell, 1989): an N-terminal catalytic region, which binds molybdopterin and is similar to human sulphite oxidase; a central region, which is a cytochrome *b*₅₅₇ sequence that binds haem; and a C-terminal region, which binds FAD and a reduced pyridine nucleotide and is a cytochrome *b* reductase (CbR).

Eukaryotic assimilatory NaRs form three classes based upon which reduced pyridine nucleotide they oxidize

(Kinghorn & Campbell, 1989). *Aspergillus nidulans*, in common with other fungi, has an NADPH-specific NaR (NADPH: nitrate oxidoreductase, EC 1.6.6.3). Higher plants usually contain an NADH-specific NaR (EC 1.6.6.1), which has also been found in some algae. A third type, NAD(P)H-bispecific NaR (EC 1.6.6.2), has been found in some higher plants and algae, but is rarely the sole NaR in these organisms (Friemann *et al.*, 1991).

To examine co-enzyme specificity, Friemann *et al.* (1991) aligned birch bispecific NaR with NADH- and NADPH-specific NaRs. They concluded that in NADH-specific enzymes, three successive proline residues are conserved whilst only one is conserved in the birch enzyme; a different proline residue is conserved in NADPH-specific enzymes. The importance of this sequence was tested in birch NaR by site-directed mutagenesis, which demonstrated that the NADPH-dependent activity of the bispecific enzyme could be reduced (to 7%) by replacing the proline residues which are missing in the wild-type

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Abbreviations: CbR, cytochrome *b* reductase; G6PD, glucose-6-phosphate dehydrogenase; NaR, nitrate reductase; PPP, pentose phosphate pathway.

birch enzyme; however, NADH-dependent activity was also reduced by 50% (Shondorf & Hachtel, 1995). A similar study in *A. nidulans*, however, did not alter the specificity or activity of NaR at all (Garde *et al.*, 1995), suggesting that other residues must be involved.

The three-dimensional structure of the C-terminal domain of corn NaR has now been deduced by X-ray crystallography (Lu *et al.*, 1994, 1995; Campbell, 1996), demonstrating that this CbR fragment is a member of the FNR family of flavoenzymes, named from ferredoxin NADP⁺ reductase (Karplus *et al.*, 1991). Whilst members of this family have little overall amino acid similarity, their three-dimensional structures in the NADH/NADPH domains are very similar, that is a variation of the Rossmann or dinucleotide fold found in many dehydrogenases and reductases (see Shiraishi *et al.*, 1998). The fine structure of the binding pocket for the ribose 2' hydroxyl of the adenine of NADH and 2' phosphate of NADPH has been defined for some FNR family members (Campbell, 1996; and see Shiraishi *et al.*, 1998). The presence of a negatively charged side-chain, such as aspartic acid, at the end of the third β -strand of this pocket is characteristic of NAD⁺/NADH-specific enzymes (residue 205 in Lu *et al.*, 1994), whereas NADP⁺/NADPH-specific enzymes, including *A. nidulans* NaR, have serine or threonine at this position (see Shiraishi *et al.*, 1998). Equivalent residues were identified as being involved in cofactor specificity in glutathione reductase (Scrutton *et al.*, 1990).

It is only recently that a mutation at this position (the S920D mutation of *Neurospora crassa*) has been induced (Shiraishi *et al.*, 1998), but this was studied only as the CbR fragment of this enzyme expressed in *Escherichia coli*. In the wild-type, NADPH-dependent activity was 113-fold greater than the NADH-dependent activity. Substitution of this serine by aspartic acid reduces electron donation by NADPH to 2.4% of the wild-type. Concomitantly, NADH-dependent activity was increased by 15-fold relative to the NADH-dependent activity in the wild-type; however, this only represents 13.5% of the wild-type NADPH-dependent activity, and is only 5.6 times the NADPH-dependent activity of this mutant. Whilst the NADPH/NADH specificity had essentially been reversed, the levels of activity clearly show that other residues are involved.

Such studies of NaR fragments in heterologous systems are ideal for kinetic analyses of the enzyme. However, they do not allow investigation of the activity of the holoenzyme, of the impact of pyridine nucleotide alterations on the physiology of the organism, nor of any pleiotropic effects on gene regulation. For the ideal functional genomics approach, site-directed mutations of holoenzyme must be integrated back into the native locus in a host genome lacking any residual gene sequences: this prevents spurious genomic effects from duplicate sequences (either mutant or wild-type) and heterologous dimerization between wild-type and mutant proteins. *A. nidulans* offers such a system (Garde *et al.*, 1995). In this fungus, the structural genes for NaR

(*niaD*) and nitrite reductase (*niiA*) are tightly linked and are divergently transcribed (Tomsett & Cove, 1979; Johnstone *et al.*, 1990); transformation of a deletion strain allows selection for *niiA*⁺ arising from homologous targeted integration (Garde *et al.*, 1995).

In *A. nidulans*, NaR expression is regulated both by substrate induction via the *nirA* gene product and by nitrogen metabolite repression mediated by ammonium/glutamine and the *areA* gene product (see Crawford & Arst, 1993). Furthermore, the interaction between NaR and NAD(P)H has been implicated in the regulation of the pentose phosphate pathway (PPP) and in the post-transcriptional regulation of NaR activity (Dunn-Coleman & Pateman, 1977; and see Cove, 1979). Hankinson & Cove (1974) have demonstrated that the enzymes of the PPP are induced in the presence of nitrate; this has also been observed in plants (Redinbaugh & Campbell, 1998). This induction could occur via the *nirA* pathway or via another pathway which detects the NADPH/NADP⁺ ratio in the cell, causing induction when NaR is active and NADPH is oxidized. Most evidence points to the former of these two models, as constitutive *niaD* mutants are constitutive for NADPH synthesis. However, the latter theory cannot be ruled out as these constitutive mutants may retain diaphorase activities, which could induce the PPP by oxidizing NADPH.

A. nidulans and *N. crassa* NaRs have also been shown to be protected from deactivation in the presence of NADPH, and deactivation can be reversed by subsequent incubation with NADPH; NADH cannot protect NaR in these organisms (Subramanian & Sorger, 1972; Dunn-Coleman & Pateman, 1977). The mechanism for this deactivation and subsequent reactivation is not understood.

Here, the effect of mutations altering pyridine nucleotide specificity, created by site-directed mutagenesis and reintroduced back into a *niaD* deletion strain using a homologous transformation system, have allowed analysis of their effect not only on NaR catalytic activity, but also on the growth and metabolism of *A. nidulans*, in particular the regulation of the PPP.

METHODS

***A. nidulans* strains and phenotypic studies of mutants.** The strains, media, growth conditions and phenotypic tests used in this study were as described previously (Cove, 1966; Tomsett & Cove, 1979; Garde *et al.*, 1995). The *niaD* mutations created by Garde *et al.* (1995) are referred to here as follows: *niaD3004* (*niaDE850P*), *niaD3005* (*niaDEA850/1PP*), *niaD3010* (*niaDH654A*). The mutations created in this study (*niaD3000*, 3001, 3002, 3006, 3007, 3008, 3009) are defined in Fig. 1.

Site-directed mutagenesis and vector construction. PCR was carried out on a cloned fragment of the *niaD* gene using a mutagenic primer (i.e. containing mismatch bases) to introduce two mutations: T813D, K814Q. This PCR product was eluted from an agarose gel and combined with a second PCR product by recombinant PCR (Vallette *et al.*, 1989) to

Corn NADH:NaR	V	I	D	Q	V	K	//	G	P	P	P	M
	gtc	atc	gac	cag	gta	aag	//	gga	cca	ccg	ccg	atg
<i>N. crassa</i> NADPH:NaR	T	L	S	R	P	G	//	G	P	E	G	M
	act	ttg	agt	aga	ccg	ggt	//	ggg	ccg	gag	gga	atg
<i>A. nidulans</i> NADPH:NaR (wild-type <i>niaD</i>)	T	L	T	K	G	S	//	G	P	E	A	M
	aca	ctg	acg	aag	ggg	agt	//	ggg	cct	gag	gcg	atg
<i>niaD3000</i>	T	L	D	Q	G	S	//	G	P	E	A	M
	aca	ctg	gac	cag	ggg	agt	//	ggg	cct	gag	gcg	atg
<i>niaD3001</i>	T	L	D	Q	G	S	//	G	P	P	A	M
	aca	ctg	gac	cag	ggg	agt	//	ggg	cct	ccg	gcg	atg
<i>niaD3002</i>	T	L	D	Q	G	S	//	G	P	P	P	M
	aca	ctg	gac	cag	ggg	agt	//	ggg	cct	ccg	ccg	atg
Revertant <i>niaD3006</i>	T	L	N	Q	G	S	//	G	P	P	A	M
	aca	ctg	<u>aac</u>	cag	ggg	agt	//	ggg	cct	ccg	gcg	atg
Revertant <i>niaD3007</i>	T	L	I	Q	G	S	//	G	P	P	A	M
	aca	ctg	<u>att</u>	cag	ggg	agt	//	ggg	cct	ccg	gcg	atg
Revertant <i>niaD3008</i>	T	L	V	Q	G	S	//	G	P	P	A	M
	aca	ctg	<u>gtc</u>	cag	ggg	agt	//	ggg	cct	ccg	gcg	atg
Revertant <i>niaD3009</i>	T	L	G	Q	G	S	//	G	P	P	A	M
	aca	ctg	<u>ggc</u>	cag	ggg	agt	//	ggg	cct	ccg	gcg	atg

Fig. 1. Comparison of *A. nidulans* wild-type (GenBank accession no. M58291), mutant and revertant *niaD* gene sequences and the resulting NaR amino acid sequence with the equivalent sequences from corn (AF153448) and *N. crassa* (X61303.1). Sequences are shown only in the region of interest. The amino acid sequence is shown in upper-case letters and the DNA sequence in lower case. The DNA and amino acid sequence which is altered from the wild-type is shown in bold, and the sequence which is altered in the revertants with respect to the original mutation is underlined. The gap between the two mutation sites is denoted by two slashes.

create a 472 bp mutant fragment. This fragment was cloned into three vectors (pUC/*niaD*/KK, pUC/*niaD*/KK/E850P and pUC/*niaD*/KK/EA850/1PP), each containing a *KpnI* fragment from the *niaD* gene, one wild-type and two containing mutations created by Garde *et al.* (1995). The PCR fragments were cloned in at *NcoI* and *SacII* sites. The *KpnI* fragments were subcloned into the transformation vector pUC/5'*niiA*/*niaD* (Garde *et al.*, 1995). All cloning was carried out using the methods of Sambrook *et al.* (1989). To ensure that no PCR errors were introduced into the gene and to check for the presence of the desired mutations, the area of interest was sequenced using an ABI 373 automated sequencer (Applied Biosystems) using the manufacturer's instructions.

Protoplast preparation and transformation. Protoplasts of *A. nidulans* strain *niiA niaDΔ503 (pabaA1)* (Tomsett & Cove, 1979) were made by the method of Johnstone *et al.* (1990), including modifications of Garde *et al.* (1995). DNA (5 µg) was added to 100 µl protoplasts in a 0.4 mm electroporation cuvette and incubated on ice for 1 min. The protoplasts were then transformed by electroporation (Garde *et al.*, 1995). The protoplast suspension was plated onto supplemented minimal medium (Cove, 1966) containing 1.2 M sorbitol and 10 mM potassium nitrite to select for *niiA*⁺ transformants. Transformant homokaryons were purified twice by streaking single

colonies onto selective medium. DNA was isolated using the method of Tilburn *et al.* (1983). The genomic DNA was cut with *Sall*, and Southern blotting (Southern, 1975) was performed onto ZetaProbe (Bio-Rad) membrane following the manufacturer's protocol to ensure that only single-copy transformants were studied.

Enzyme assays. Cultures (200 ml) were inoculated with 2×10^6 fresh conidia and grown by shaking for 17 h at 30 °C in liquid minimal medium with supplements and 10 mM urea as the nitrogen source. Induced cultures also contained 20 mM potassium nitrate. The mycelium was strained, washed through muslin and cell-free extracts produced by the method of MacDonald & Cove. (1974). The mycelium was extracted in fresh 0.1 M sodium phosphate buffer pH 7.2, 5 mM EDTA, 5 mM cysteine, 10% (v/v) glycerol for the nitrite reductase assay, and 0.1 M sodium phosphate buffer pH 6.8, 1 mM β-mercaptoethanol, 0.5 mM EDTA, 1% (w/v) NaCl for the NaR assay and its partial activity assays. Nitrite reductase activity was measured as the oxidation of NADPH by a decrease in absorbance at 340 nm over 4 min as described by Pateman *et al.* (1967). NaR activity was measured as the production of NO₂⁻ by an increase in absorbance at 340 nm according to Garrett & Cove (1976). Reduced methyl violet and bromophenol blue partial activities of NaR were

measured as described by Garrett & Nason (1969) and Campbell (1986), respectively. NaR was assayed in triplicate and nitrite reductase in duplicate; all assays were carried out over two or more reaction times that gave a linear response. The glucose-6-phosphate dehydrogenase (G6PD) assay was adapted slightly from Hankinson & Cove (1974). Protein was extracted as previously described in 50 mM Tris/HCl pH 7.4. The assay mixture, made up in 1 ml quartz cuvettes, contained 50 µl glucose 6-phosphate (disodium salt), 50 µl 12 mM NADP⁺ (disodium salt) and 50 or 20 µl of cell-free extract, and was made up to 1 ml with Tris/HCl pH 8.0. In each case it was checked that there was negligible activity before glucose 6-phosphate was added, then the initial rate at 340 nm was determined. The activity was measured as the rate of reduction of NADP⁺ to NADPH. Protein concentrations were measured using the Bradford (1976) method.

UV-induced reversion of *A. nidulans* conidia. Conidial suspensions [10^8 conidia ml⁻¹ in a 1% (v/v) Tween 80 solution] were exposed to 1200 J m⁻² UV radiation (to achieve 5% survival) whilst shaking under a UV lamp, and then plated onto appropriate nitrate-selective media allowing only revertant colonies to grow. DNA was extracted from revertants (as above) and a region of *niaD* was amplified by PCR and sequenced using an ABI 370 automated sequencer (Applied Biosystems) using the manufacturer's recommended protocol.

RESULTS AND DISCUSSION

Production and characterization of mutants with altered pyridine nucleotide specificities

Alignment of the sequence of *A. nidulans* NaR with other nitrate reductases indicated that T813 and K814 are in equivalent positions to those residues (D205/Q206 in corn leaf; S920/R921 in *N. crassa*) implicated in conferring NADH/NADPH specificity (Lu *et al.*, 1994; Shiraishi *et al.*, 1998). Three mutant genes were created by site-directed mutagenesis and subcloning (Fig. 1). The first, *niaD3000*, incorporated the putative NADH-specific 'DQ' sequence from corn. The other two, *niaD3001* and *niaD3002*, combined these mutations with changes to the prolines described earlier (Friemann *et al.*, 1991; Garde *et al.*, 1995; Shondorf & Hachtel, 1995). The plasmids were transformed into *A. nidulans* and the genomic DNA of a single transformant from each transformation was extracted and Southern-blotted to ensure each contained a single homologous integration of the pUC5'*niiA/niaD* plasmid.

To characterize these mutants, the activities of a series of enzymes were measured: NADPH:NaR and NADH:NaR for activity of the holoenzyme; partial activities for mutant NaR using the artificial electron donors bromophenol blue and methyl viologen, which transfer electrons to the cytochrome and molybdopterin regions of the protein, respectively (see Solomonson & Barber, 1990); and NADPH nitrite reductase, the second enzyme of the pathway which is co-regulated with NaR. As shown in Table 1, a very low background NADPH:NaR activity was detected in all single or double mutant strains (*niaD3000*, *3001*, *3002*) containing the T813D, K814Q substitutions. Bromophenol blue:NaR activity levels in these mutants were normal,

indicating that the mutation affected only the CbR region of the enzyme. In contrast, the *niaD3004* (E850P) and *niaD3005* (E850P, A851P) mutants displayed wild-type levels of all enzyme activities, as described previously (Garde *et al.*, 1995). All mutants were inducible for NADPH:NiR activity.

NADH:NaR was also measured to detect whether the co-enzyme specificity had altered. Like the wild-type, the *niaD3004* and *niaD3005* mutants had essentially no NADH:NaR activity. In all strains carrying the T813D, K814Q mutation, the level of activity had increased 40-fold relative to the wild-type NADH-dependent activity and was approximately 33% of induced NADPH:NaR activity in the wild-type. There was no significant difference between any of the single or double T813D, K814Q substitution strains, indicating that this mutation alone determines the observed alteration in co-enzyme specificity. This was consistent with the data of Shiraishi *et al.* (1998), indicating that an acidic side-chain at this position is crucial for NADH specificity of NaR.

The phenotype of each mutant was tested by growth on a range of nitrogen source and chlorate media (data not shown). The *niaD3004* and the *niaD3005* mutants displayed wild-type phenotypes on all media as expected (Garde *et al.*, 1995). However, all single or double mutant strains (*niaD3000*, *3001*, *3002*) containing the T813D, K814Q mutation were unable to utilize nitrate as sole N source and displayed wild-type sensitivity to chlorate. This inability to utilize nitrate cannot be due to lack of expression or reduced synthesis of the NiaD protein because significant NADH:NaR activity was detected *in vitro*. The specific activity of the NADH:NaR in these strains [39–45 nmol NO₂ min⁻¹ (mg protein)⁻¹] was more than sufficient to support growth. Garde *et al.* (1995) demonstrated that the *niaD3010* (H654A) mutant grew normally on nitrate as sole nitrogen source, despite having an NADPH:NaR with a specific activity of only 28 nmol NO₂ min⁻¹ (mg protein)⁻¹. For this reason, these strains were not tested by Northern or Western analysis. This suggests that the lack of growth on nitrate occurs for other reasons: a metabolic effect *in vivo*, for example NADPH/NADH competition or insufficient NADH pools to drive nitrate assimilation in addition to other cellular reactions; a conformational effect *in vivo* which reduces catalytic efficiency, for example the pyridine nucleotide pocket for NADH is not optimal, but *in vitro* this is overcome by high concentrations of NADH; a regulatory effect, for example post-transcriptional inactivation/phosphorylation; or a toxicity effect arising from increased levels of NADPH in the mutant background.

Is lack of growth on nitrate due to NADPH competition *in vivo*?

If the mutant enzyme was binding but not oxidizing NADPH *in vivo*, NADH may be excluded; whereas in standard *in vitro* assays, NADPH concentrations would

Table 1. Enzyme activities of wild-type, mutant and revertant strains

NADPH:NaR activity, NADH:NaR activity and the partial activities bromophenol blue (BPB):NaR and methyl viologen (MV):NaR were assayed in triplicate for each strain after culturing under inducing (I) or uninducing (U) conditions. Means and standard deviations of the specific activities are shown. All NaR activities are expressed in nmol NO₂ produced min⁻¹ (mg protein)⁻¹. NADPH:nitrite reductase (NiR) activity was measured in duplicate and is expressed in nmol NADPH oxidized min⁻¹ (mg protein)⁻¹. ND, Not determined.

Strain	Mutations	NADPH:NaR		NADH:NaR		BPB:NaR		MV:NaR		NADPH:NiR	
		I	U	I	U	I	U	I	U	I	U
Wild-type	–	131±12	8±1	0±2	1±1	62±11	2±0	59±2	5±3	32	5
<i>niaD3000</i>	T813D, K814Q	2±1	2±1	41±5	2±1	72±8	1±2	61±1	3±2	49	3
<i>niaD3001</i>	T813D, K814Q, E850P	2±1	5±0	39±4	3±1	81±5	2±3	64±5	1±3	54	2
<i>niaD3002</i>	T813D, K814Q, E850P, A851P	1±1	1±1	45±4	3±1	65±9	2±2	61±8	0±0	51	4
<i>niaD3004</i>	E850P	120±7	7±3	1±0	1±0	71±7	0±0	55±6	2±4	37	2
<i>niaD3005</i>	E850P, A851P	124±15	7±1	2±1	2±1	67±10	0±1	49±3	2±1	41	3
<i>niaD3006</i>	T813N, K814Q, E850P	85±10	4±2	1±0	0±0	ND	ND	ND	ND	ND	ND
<i>niaD3007</i>	T813I, K814Q, E850	92±12	3±1	0±0	2±1	ND	ND	ND	ND	ND	ND
<i>niaD3008</i>	T813V, K814Q, E850P	61±14	3±3	0±0	0±1	ND	ND	ND	ND	ND	ND
<i>niaD3009</i>	T813G, K814Q, E850P	55±7	3±1	1±0	0±0	ND	ND	ND	ND	ND	ND
<i>niaDΔ503</i>	Deletion of entire <i>niaD</i> gene	0±1	0±0	0±0	0±0	0±0	0±0	0±0	0±0	62	57

Table 2. Half-lives (min) of wild-type and mutant enzymes when incubated in the presence and absence of NADPH and NADH

Extracts were incubated at 37 °C in the presence or absence of either NADPH or NADH at a concentration of 2 mM. The two controls had 2 mM of the appropriate co-enzyme added just prior to assay. Activity was measured at 0, 6, 12, 20, 30 and 42 min for each sample.

Strain	Mutations	+ NADPH	– NADPH	+ NADH	– NADH
Wild-type	–	> 42*	8.0	7.5	7.8
<i>niaD3000</i>	T813D, K814Q	3.1	3.3	3.0	3.7
<i>niaD3001</i>	T813D, K814Q, E850P	3.2	4.5	4.9	4.9
<i>niaD3002</i>	T813D, K814Q, E850P, A851P	3.8	3.7	4.2	4.0

* No significant change in activity was observed for this strain over the course of the experiment.

be too low. This hypothesis was tested in a competition assay. The mutant extracts were tested for activity in comparison with the wild-type, both with a varying amount of NADPH against a fixed amount of NADH and vice versa. In both strains, activity decreased slightly as the total NADPH/NADH concentration increased, but there was no evidence for competition (data not shown).

Dunn-Coleman & Pateman (1977) have shown that whilst NADPH protects wild-type NaR from deactivation in cell-free extracts, NADH does not. Using similar protection assays (Table 2), the half-life of the wild-type enzyme was > 42 min when incubated with NADPH, but was around 8 min in the absence of any reduced cofactor or in the presence of NADH. However, all of the mutant enzymes had half-lives of 3–5 min in the presence and absence of NADPH and NADH. This indicates that NADPH is specific for protection of NaR

against deactivation and that NADH cannot substitute in the mutant NADH-specific NaR. The mechanism of this protection is unknown. If catalytic, it is possible that a conformational change results from binding of the phosphate group of NADPH to the amino acids T813 and K814. However, a regulatory response triggered by NADPH oxidation is also possible.

Since NaR and NAD(P)H have been implicated in the regulation of the PPP (see Cove, 1979), these mutants were tested for G6PD activity (Fig. 2). All strains showed induction. The increase in activity of G6PD was about threefold for each strain. This would appear to rule out an effect of the NADPH/NADP⁺ ratio in the regulation of the PPP since the T813D, K814Q (*niaD3000*, *3001*, *3002*) NaR enzyme cannot oxidize NADPH, and since the mutant does not grow on nitrate, there is insufficient NADPH oxidation from NADPH:NiR. Rather, it suggests that nitrate induces the PPP via the *nirA* gene

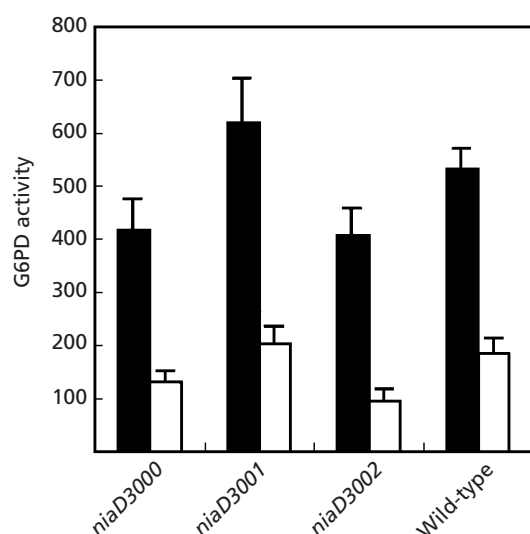


Fig. 2. Nitrate-induced (■) and uninduced (□) activities of G6PD in wild-type and T813D, K814Q substitution mutants. Activity is expressed in nM NADP⁺ reduced min⁻¹ (mg total protein)⁻¹. Data are means and standard deviations of three separate experiments.

product, in much the same way as the enzymes of nitrate assimilation are induced.

Reversion analysis of *niaD3001*

As the T813D, K814Q mutants did not grow on nitrate, it was possible to select UV-induced revertants by growth on nitrate as the sole nitrogen source. These could arise from mutations within the *niaD* gene which optimize the interaction of NADH with the enzyme or restore NADPH:NaR activity. Alternatively, mutations could be selected in other genes which, for example, increased the available pool of NADH *in vivo* or disengaged a regulatory inactivation of the enzyme. Fourteen revertants of the *niaD3001* mutant were isolated and their *niaD* gene was sequenced in the region spanning the primary mutations. In growth tests, all were indistinguishable from the wild-type (data not shown).

All revertants contained nucleotide alterations (mostly single base changes) which substituted the single amino acid residue D813; an acidic residue at this position is thus responsible for elimination of growth on nitrate. No alterations occurred at the Q814 site or at P850. Four classes of revertant were identified (Fig. 1); all amino acid substitutions replaced the negatively charged aspartic acid residue with an uncharged amino acid (I, V, G, N). This further supports the view that it is principally the presence of an acidic residue at this position that provides specificity to NADH:NaR (Shiraishi *et al.*, 1998). Conclusive proof will require the reciprocal experiment on an NADH-specific NaR to show that replacement of the acidic residue by a

small neutral residue also destroys NADH specificity (Shiraishi *et al.*, 1998).

In enzyme assays, none of the revertants had NADH activity, one from each class was analysed in detail (Table 1). Substitution of the aspartic acid residue restored NADPH:NaR activity to levels capable of supporting normal growth: asparagine 65 % of wild-type; glycine 42 %; isoleucine 70 %; valine 47 %.

Reversion of the *niaD3000* and *niaD3002* mutants produced no new classes (data not shown). The reversion frequencies for each strain were almost identical (data not shown), suggesting that the number of possible reversions were the same for each mutation; i.e. the alterations at E850 and A851 did not affect the reversion frequency and hence were not involved in preventing enzyme activity or restoring it.

The NAD(P)H specificity of NaR

Site-specific mutagenesis demonstrated that the T813D, K814Q mutation alters co-enzyme specificity and that the E850P and A851P substitutions had no effect, even in a *niaD3000* background. This would seem to contradict the evidence from birch (Shondorf & Hachtel, 1995). The removal of the acidic side-chain at position 813 was sufficient to eliminate NADH as electron donor and restore NADPH-dependent activity. Since a series of isolates all had this change, this suggests that either a more complex change is required to mutate *niaD* to increase the efficiency of NADH utilization, or that lack of growth on nitrate is a metabolic or regulatory control (see below). The fact that the mutant enzymes had significant NADH:NaR *in vitro* suggests that although subsequent alterations to the CbR region of this enzyme may be able to further optimize the NaR/NADH interaction, perhaps through modification to the size/shape of the binding pocket, these changes may not be detected in the reversion analysis of *niaD3001* as they would not restore growth. The lack of growth is probably not due to a lack of NADH:NaR activity but is most likely caused by other factors (see below).

Site-directed mutagenesis of the equivalent sequence has been undertaken on *N. crassa* NADPH:NaR to examine pyridine nucleotide specificity. Gonzalez *et al.* (1995) showed that a serine to alanine (S920A) substitution reduced NADPH:NaR activity by 40 % when transformed back into *N. crassa*, whilst a S920T substitution increased activity by 75 %. The latter observation is not surprising since *A. nidulans* has threonine at this position (T813), except that it indicates that *N. crassa* may have evolved a suboptimal NaR activity.

Is lack of growth on nitrate due to NADH limitation or altered post-transcriptional regulation?

The strains carrying the NADH:NaR enzyme do not grow on nitrate, which would not be expected from the enzyme assay results. It has been shown that mutants with 20 % wild-type NADPH-dependent activity still grow well on nitrate (Garde *et al.*, 1995). It could be that

NADH is, for some reason, not available to NaR *in vivo*; however, this seems unlikely as it has been demonstrated in *A. niger* that NADH is abundant in cells and that levels are maintained even when it is in demand (Fuhrer *et al.*, 1980). It would therefore seem likely that whilst the mutant enzyme is able to bind and oxidize NADH in cell-free extracts, *in vivo* conditions prevent this.

NADH and NADPH have also been implicated in the post-translational regulation of NaR activity in a number of different organisms (Minagawa & Yoshimoto, 1982; Dunn-Coleman & Pateman, 1977; Vega *et al.*, 1975; Solomonson *et al.*, 1973). The mechanism by which NADPH stabilizes NaR is not known but it would seem likely that the binding of the co-enzyme induces a conformational change that protects the enzyme from cellular factors that deactivate it. Dunn-Coleman & Pateman (1977) showed that the deactivation is reversible, so it would seem likely that the deactivation occurs via a phosphorylation event rather than by proteolysis. Lillo & Ruoff (1992), working on *Cucurbita maxima*, suggested that this deactivation may occur at a second binding site, as the protection could occur with NADH or NADPH, whilst only NADH could bind to the catalytic binding site of the enzyme. This work presents evidence that protection occurs via binding of NADPH to the catalytic binding site as disruption of this site prevents NADPH protection. Also, the fact that the mutant enzymes have a shorter half-life than the wild-type in the absence of reducing cofactor would suggest that the mutation must affect the inherent stability of the enzymes.

Could increased levels of NADPH in the mutant background be toxic?

The results shown in Fig. 2 demonstrated that the PPP was induced in the mutant strains just as in the wild-type; however NADPH, its product, would not have been oxidized by the altered NaR enzyme. It is thus possible that increased levels of NADPH in these strains could be toxic and hence could be responsible for the inability of the mutants to grow on nitrate. However, the mutants had a nitrogen-starved morphology when grown on nitrate as the sole nitrogen source, perhaps suggesting that NADPH toxicity was not the cause of this phenotype. In an attempt to test for NADPH toxicity, a *niaD3001* mutant was crossed into a strain carrying the *pppA1* mutation in the G6PD enzyme of the PPP (Hankinson & Cove, 1974). Preliminary data suggested that the double mutant showed slightly increased growth on nitrate compared to the *niaD3001* mutant alone (data not shown). This suggested that whilst NADPH may be slightly toxic, it is not the principal factor causing the inability of the *niaD3001* mutant to grow on nitrate.

This study has demonstrated the importance of functional genomic analysis *in vivo*; investigations of an enzyme function must analyse changes to the physiology of the organism within which it has evolved.

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REFERENCES

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Campbell, W. H. (1986). Properties of bromophenol blue as an electron-donor for higher-plant NADH-nitrate reductase. *Plant Physiol* **82**, 729–732.
- Campbell, W. H. (1996). Nitrate reductase biochemistry comes of age. *Mol Gen Genet* **111**, 355–361.
- Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim Biophys Acta* **113**, 51–56.
- Cove, D. J. (1979). Genetic studies of nitrate assimilation in *Aspergillus nidulans*. *Biol Rev* **54**, 291–327.
- Crawford, N. M. & Arst, H. N., Jr (1993). The molecular genetics of nitrate assimilation in plants. *Annu Rev Genet* **27**, 115–146.
- Dunn-Coleman, N. S. & Pateman, J. A. (1977). *In vivo* and *in vitro* studies of nitrate reductase in *Aspergillus nidulans*. *Mol Gen Genet* **152**, 285–293.
- Friemann, A., Brinkmann, K. & Hachtel, W. (1991). Sequence of a cDNA encoding the bi-specific NAD(P)H-nitrate reductase from the tree *Betula pendula* and identification of conserved protein regions. *Mol Gen Genet* **227**, 97–105.
- Fuhrer, L., Kubicek, C. P. & Rohr, M. (1980). Pyridine nucleotide levels and ratios in *Aspergillus niger*. *Can J Microbiol* **26**, 405–408.
- Garde, J., Kinghorn, J. R. & Tomsett, A. B. (1995). Site-directed mutagenesis of nitrate reductase from *Aspergillus nidulans*. Identification of some essential and some nonessential amino acids among conserved residues. *J Biol Chem* **270**, 6644–6650.
- Garrett, R. H. & Cove, D. J. (1976). Formation of NADPH-nitrate reductase activity *in vitro* from *niaD* and *cnx* mutants. *Mol Gen Genet* **149**, 189–196.
- Garrett, R. H. & Nason, A. (1969). Further purification and properties of *Neurospora crassa* nitrate reductase. *J Biol Chem* **244**, 2870–2882.
- Gonzalez, C., Brito, N. & Marzluf, G. A. (1995). Functional analysis by site-directed mutagenesis of individual amino acid residues in the flavin domain of *Neurospora crassa* nitrate reductase. *Mol Gen Genet* **249**, 456–464.
- Hankinson, O. & Cove, D. J. (1974). Regulation of the pentose phosphate pathway in the fungus *Aspergillus nidulans*. *Can J Microbiol* **21**, 99–101.
- Johnstone, I. L., McCabe, P. C., Greaves, P. & 7 other authors (1990). Isolation and characterisation of the *crnA-niiA-niaD* gene cluster for nitrate assimilation in *Aspergillus nidulans*. *Gene* **90**, 181–192.
- Karplus, P. A., Daniels, M. J. & Herriott, J. R. (1991). Atomic structure of ferredoxin:NADP⁺ reductase: prototype for a structurally novel flavoenzyme family. *Science* **251**, 60–66.
- Kinghorn, J. R. & Campbell, E. I. (1989). Amino acid sequence comparisons of nitrate reductases. In *Molecular and Genetic Aspects of Nitrate Assimilation*, pp. 385–403. Edited by J. L. Wray & J. R. Kinghorn. Cambridge: Cambridge University Press.

- Lillo, C. & Ruoff, P. (1992). Hysteretic behavior of nitrate reductase. Evidence of an allosteric binding site for reduced pyridine nucleotides. *J Biol Chem* **267**, 13456–13459.
- Lu, G., Campbell, W. H., Schneider, G. & Lindqvist, Y. (1994). Crystal structure of the FAD-containing fragment of corn leaf nitrate reductase at 2.5 Å resolution: relationship to other flavoprotein reductases. *Structure* **2**, 809–821.
- Lu, G., Lindqvist, Y., Schneider, G., Dwivedi, U. N. & Campbell, W. H. (1995). Structural studies on corn nitrate reductase: refined structure of the cytochrome *b* reductase fragment at 2.5 Å, its ADP complex and an active site mutant and modeling of the cytochrome *b* domain. *J Mol Biol* **248**, 931–948.
- MacDonald, D. W. & Cove, D. J. (1974). Studies on temperature sensitive mutants affecting the assimilatory nitrate reductase of *Aspergillus nidulans*. *Eur J Biochem* **46**, 169–178.
- Minagawa, N. & Yoshimoto, A. (1982). The *in vitro* irreversible inactivation by NAD(P)H of assimilatory nitrate reductase from *Hansenula anomala*. *Agric Biol Chem* **49**, 2213–2215.
- Pateman, J. A., Rever, B. M. & Cove, D. J. (1967). Genetic and biochemical studies of nitrate reduction in *Aspergillus nidulans*. *Biochem J* **104**, 103–111.
- Redinbaugh, M. G. & Campbell, W. H. (1998). Nitrate regulation of the oxidative pentose phosphate pathway in maize (*Zea mays* L.) root plastids: induction of 6-phosphogluconate dehydrogenase activity, protein and transcript levels. *Plant Sci* **134**, 129–140.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Scrutton, N. S., Berry, A. & Perham, R. N. (1990). Redesign of the co-enzyme specificity of a dehydrogenase by protein engineering. *Nature* **343**, 38–41.
- Shiraishi, N., Croy, C., Kaur, J. & Campbell, W. H. (1998). Engineering of pyridine nucleotide specificity of nitrate reductase: mutagenesis of recombinant cytochrome *b* reductase fragment of *Neurospora crassa* NADPH:nitrate reductase. *Arch Biochem Biophys* **358**, 104–115.
- Shondorf, T. & Hachtel, W. (1995). The choice of reducing substrate is altered by replacement of an alanine by a proline in the FAD domain of a bispecific NAD(P)H-nitrate reductase from birch. *Plant Physiol* **108**, 203–210.
- Solomonson, L. P. & Barber, M. J. (1990). Assimilatory nitrate reductase: functional properties and regulation. *Annu Rev Plant Phys Plant Mol Biol* **41**, 225–253.
- Solomonson, L. P., Jetschmann, K. & Vennesland, B. (1973). Reversible inactivation of the nitrate reductase of *Chlorella vulgaris* Beijerinck. *Biochim Biophys Acta* **309**, 32–43.
- Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.
- Subramanian, K. N. & Sorger, G. J. (1972). Regulation of nitrate reductase in *Neurospora crassa*: stability *in vivo*. *J Bacteriol* **110**, 538–546.
- Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabickyzissman, J. H., Lockington, R. A. & Davis, R. W. (1983). Transformation by integration in *Aspergillus nidulans*. *Gene* **26**, 205–221.
- Tomsett, A. B. & Cove, D. J. (1979). Deletion mapping of the *niiA niaD* gene region of *Aspergillus nidulans*. *Genet Res* **34**, 19–32.
- Vallette, F., Mege, E., Reiss, A. & Adesnik, M. (1989). Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Res* **17**, 723–733.
- Vega, J. M., Greenbaum, P. & Garrett, R. (1975). Studies on the inactivation of the *Neurospora crassa* assimilatory nitrate reductase in the presence of reduced pyridine nucleotides plus flavin. *Biochim Biophys Acta* **377**, 251–257.

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