Further characterization of the assimilatory nitrate reductase from the yeast *Candida nitratophila*

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Nitrate reductase from the yeast *Candida nitratophila* was found to contain one molecule of cytochrome b_{557} and one atom of molybdenum per subunit. FAD/haem-dependent diaphorase activity (haem domain) was associated with a 40 kDa tryptic fragment of the subunit. The 50 amino-terminal residues of this fragment were determined, and the sequence did not show significant similarity to deduced sequences of other nitrate reductases previously published. Increasing ionic strength *in vitro* had a stimulatory effect on enzymic activity via stimulation of the molybdenum-dependent terminal nitrate-reducing activity. Stimulation of activity by exogenous protein (bovine serum albumin or casein) also appeared to be an ionic effect. Stimulation of catalytic activity by phosphate was a separate effect.

Introduction

Assimilatory nitrate reductase (NR) catalyses the reduction of nitrate to nitrite in certain eukaryotic microorganisms. It has been purified and characterized from several autotrophic sources and it is clear that these NR proteins share similar physical and chemical properties (Solomonson & Barber, 1990). In addition, the availability of sequences for the NR structural genes from *Aspergillus nidulans* (Johnstone *et al.*, 1990) and *Neurospora crassa* (Okamoto *et al.*, 1991) has enabled comparisons to be made between fungal and higher plant NR proteins at the deduced amino acid sequence level (Campbell & Kinghorn, 1990). Unfortunately, however, many fungal NR proteins are unstable (Horner, 1983) and have been difficult to study *in vitro*. Consequently,

there are gaps in our knowledge of their physical and biochemical properties.

Fortunately, NR from the yeast *Candida nitratophila* is relatively stable *in vitro* and this has allowed us to study the quaternary structure of the enzyme and its spectroscopic, thermodynamic and kinetic properties (Hipkin *et al.*, 1986, 1989; Hipkin, 1989; Kay *et al.*, 1990). Here, we report further characterization of this fungal enzyme, including studies of structure–function relationships and the effects of exogenous protein and ionic strength on catalytic activity.

Methods

Organism and culture. *Candida nitratophila* was obtained from the National Collection of Yeast Cultures, Norwich, UK (catalogue no. 556) and grown at 25 °C with continuous shaking in an orbital incubator in 250 ml Erlenmeyer flasks (Hipkin *et al.*, 1986) containing 100 ml of Difco Yeast Carbon Base medium (11.7 g Difco Yeast Carbon Base l⁻¹) with nitrogen supplied as 50 mM-potassium nitrate or 50 mM-ammonium chloride.

Purification of assimilatory nitrate reductase (EC 1.6.6.2). Four litres of ammonium-grown culture was harvested at late exponential phase, washed with distilled water and resuspended for 4 h in 2 l of fresh nitrate medium under growth conditions. This treatment induced high levels of NR activity in the organism. Cells were harvested, washed with distilled water and resuspended in 50 ml 50 mM-potassium phosphate buffer, pH 7.5, containing 50 µM-FAD (Buffer A). A cell-free extract was prepared from this suspension after its passage through a French press at 100 MPa. The resulting homogenate was centrifuged at 40000 g at 4 °C for 25 min and NR protein was purified from the

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Abbreviations: ATZ, anilinothiazolinone; BV, benzyl viologen; CR, cytochrome *c* reductase (partial activity of NR); DITC, *p*-phenylene diisothiocyanate; DCPIP, 2,6-dichlorophenolindophenol; NR, nitrate reductase; PTH, phenylthiohydantoin.

supernatant by a modification of the method described by Hipkin *et al.* (1986). The modifications included an initial treatment with 2% (w/v) protamine sulphate, added 50 μ l at a time to the stirred, ice-cold extract until a clarified supernatant was obtained after centrifugation at 40000 *g*. Also, the penultimate blue Sepharose affinity-column run included a 30 ml wash with a high ionic strength buffer (0.2 M-potassium phosphate buffer, pH 7.5, containing 50 μ M-FAD)) prior to elution with 0.1 mM-NADH. Typical preparations were very pure (1000–1200-fold purification) with a final specific activity of 300–400 μ mol min⁻¹ (mg protein)⁻¹. Such preparations were homogeneous by the criterion of SDS-PAGE giving one band (after staining with Coomassie Blue) corresponding to a size of 98 kDa. Electrophoresis and protein determination were done as described previously (Hipkin *et al.*, 1986).

Enzyme assays. NAD(P)H-nitrate reductase [NAD(P)H-NR] activity, NAD(P)H-cytochrome *c* reductase [NAD(P)H-CR] activity and reduced benzyl viologen-nitrate reductase (BV-NR) activity were measured as described previously (Hipkin *et al.*, 1986). NAD(P)H-NR activity was also determined by following the nitrate-dependent oxidation of NAD(P)H at 340 nm in 1.0 ml quartz cuvettes containing 50 μ mol potassium phosphate buffer, pH 7.5, 5.0 nmol FAD, 25 μ mol potassium nitrate, 2.5 mg bovine serum albumin (BSA) and 0.1 μ mol NAD(P)H. Reactions were started by the addition of 0.05–0.1 μ g of NR.

NAD(P)H-dichlorophenolindophenol reductase [NAD(P)H-DCPIP NR] was determined by following the oxidation of NAD(P)H at 340 nm in 1.0 ml cuvettes containing 50 μ mol potassium phosphate buffer, pH 7.5, 5.0 nmol FAD, 0.5 μ mol DCPIP, 0.8 μ mol NAD(P)H and 2.5 mg BSA. Reactions were started by the addition of 0.05–0.1 μ g NR.

Measurement of molybdenum in NR protein. Molybdenum was determined by atomic absorption spectrophotometry using a Pye Unicam SP9 instrument fitted with a molybdenum Varion Techtron hollow cathode lamp (5 mA). Spectrosol molybdenum standard solution (BDH) was used for the preparation of calibration standards. Molybdenum content was also measured by the colorimetric method described by Solomonson *et al.* (1975).

Proteolytic digestion of NR. Freshly purified NR protein was incubated with trypsin (5 μ g per mg NR) in buffer A for 30 min at 30 °C. Proteolysis was terminated by placing incubation mixtures on ice and adding phenylmethanesulphonyl fluoride to a concentration equivalent to 10 times the weight of the protease. Tryptic fragments generated by short-term digestions were separated by FPLC on a Mono Q HR5/5 anion-exchange column (Pharmacia) at a flow rate of 1 ml min⁻¹. Polypeptide fragments were eluted from the column with a KCl gradient consisting of an initial 35 ml linear gradient from 0 to 0.4 M followed by a second, 5 ml linear gradient from 0.4 to 1 M. The eluate was monitored at 214 nm.

Protein determination. NR protein was estimated by a modification of the method of Bradford (1975) using Bio-Rad reagent. The Bio-Rad microassay was used to determine protein concentrations in the range 5–20 μ g and the macroassay was used to determine concentrations in the range 20–100 μ g. Calibration curves were prepared using BSA and purified NR protein (dialysed against water and lyophilized) as standards.

Amino acid sequencing. Tryptic fragments, separated by SDS-PAGE and identified after staining with Coomassie Blue, were cut out of gels, electroeluted and lyophilized. The lyophilized peptide was dissolved in 0.03 ml 0.2 M-Na₂HPO₄ containing 0.25% (w/v) SDS and added to 10 mg of DITC glass (17 nm pore size, 200–400 mesh) prepared by a method modified from Watcher *et al.* (1973). The glass was incubated for 60 min at 56 °C under nitrogen, then washed with water and

methanol to remove noncovalently-bound material. The glass-coupled peptide was then sequenced by automated solid-phase Edman degradation (Laursen, 1971) using the microsequence facility built by the SERC Sequence Unit in the Department of Biochemistry, University of Leeds, UK. ATZ-amino acids were converted for 20 min at 70 °C (under nitrogen) and the corresponding PTH-amino acids identified by reverse-phase (C₁₈) HPLC, using a gradient of acetonitrile in sodium acetate buffer, pH 4.9. The PTH-amino acids were quantified at 269 nm and serine and threonine residues confirmed by detection of their dehydro-derivatives at 313 nm.

Results and Discussion

Haem and molybdenum content of yeast NR

Spectral properties of yeast NR indicate the presence of cytochrome *b*₅₅₇ as a prosthetic group (Hipkin *et al.*, 1986; Kay *et al.*, 1990). NR purified in investigations reported here had a *A*₂₈₀/*A*₄₁₃ (Soret) ratio for the oxidized enzyme in the range 2.40–2.68 and 1 mg of pure NR gave an *A*₄₁₃ of 1.05 absorbance units. Using a value for the extinction coefficient of NR cytochrome *b*₅₅₇ of 117 mM⁻¹ cm⁻¹ (Kay *et al.*, 1990), the haem content of the enzyme was calculated to be 0.9 mol haem per mol NR subunit. The molybdenum content of the enzyme estimated by atomic absorption spectroscopy was 0.71 g-atom per mol NR haem. Colorimetric analysis of a separate, purified preparation indicated a molybdenum content of 0.85 g-atom of molybdenum per mol NR haem. These results indicate the presence of one molecule of haem and one atom of molybdenum per NR subunit.

Effect of trypsin on yeast NR

NAD(P)H-NR activity was abolished after yeast NR was incubated with trypsin. However, this treatment had a differential effect on NR partial activities (Table 1). Like NAD(P)H-NR activity, BV-NR activity was inhibited strongly by this treatment but there was a stimulation of the FAD/haem-dependent diaphorase activities of the enzyme by 64% (NADH-CR) and 25% (NADH-DCPIP NR).

Separation of tryptic fragments by SDS-PAGE showed that two major peptides were produced, one of

Table 1. *Effect of tryptic digestion on NR activities*

NADH-NR activity = 11.75 μ mol NADH oxidized min⁻¹ (nmol NR)⁻¹

Treatment	Enzyme activity (%)			
	NADH-DCPIP		NADH-CR	BV-NR
	NADH-NR	NR		
Undigested	100	100	100	100
Digested	0	125	164	11

Fig. 1. Tryptic fragments of yeast NR. Yeast NR was incubated with trypsin ($5 \mu\text{g trypsin mg}^{-1}$) for 5, 10, 15, 20 or 30 min (lanes 2–6 respectively). Lane 1 shows the undigested 98 kDa subunit. The unequal staining of the two tryptic fragments shown here was not evident in other experiments.

Table 2. Amino acid composition of *Candida nitratophila* NR

Amino acid	Mol%	Amino acid	Mol%
Aspartic acid	10.52	Isoleucine	4.78
Threonine	5.40	Leucine	6.95
Serine	7.17	Tyrosine	3.43
Glutamic acid	11.16	Phenylalanine	4.61
Proline	4.72	Histidine	2.19
Glycine	12.83	Lysine	7.20
Alanine	4.50	Arginine	3.98
Valine	7.52	1/2 Cysteine	0.95
Methionine	2.10	Tryptophan	ND

ND, Not determined.

40 kDa and one of 50 kDa (Fig. 1). Products produced after a 30 min tryptic digestion were separated by FPLC, which revealed two major peaks. FPLC fractions with diaphorase activity coincided with the first major peak and those with the highest NADH-DCPIP NR activity

were pooled and immunoprecipitated with polyclonal NR antibodies (Cannons *et al.*, 1986). Electrophoresis of the immunoprecipitated product yielded a single band at 40 kDa. These results indicate that the FAD- and cytochrome b_{557} -dependent diaphorase activities of the yeast NR are associated with the 40 kDa tryptic fragment of the enzyme. Unfortunately, the small amount of tryptic fragment obtained after FPLC and immunoprecipitation did not allow us to make reliable estimates of the haem content of this peptide.

Amino acid composition and partial sequence of yeast NR

The amino acid composition of acid-hydrolysed yeast NR is shown in Table 2. In the relative composition of acidic and basic amino acids, the yeast enzyme is similar to that from higher plants (Kuo *et al.*, 1980; Redinbaugh & Campbell, 1985), algae (Giri & Ramadoss, 1979;

1 2 3 4 5 6 7 8 9 10
 Glu-Val-Val-Asp-Glu-Asp-Leu-Glu-Met-Met-
 11 12 13 14 15 16 17 18 19 20
 Ile-Asn-Pro-Glu-Lys-Arg-Asp-Val-Ile-Ile-
 21 22 23 24 25 26 27 28 29 30
 Thr-Ala-Glu-Glu-Leu-Ala-Met-His-Ala-Asn-
 31 32 33 34 35 36 37 38 38 40
 XXX-Glu-Asp-Lys-Trp-Phe-Val-Val-XXX-Pro-
 41 42 43 44 45 46 47 48 49 50
 Pro-Glu-Phe-Thr-Thr-Thr-XXX-Phe-Pro-Asp

Fig. 2. Partial amino acid sequence of the N-terminal region of the 40 kDa tryptic fragment of *C. nitratophila* NR. Residues designated XXX were not positively identified and residue identities at positions 27, 34, 35, 41, 45 and 49 (italics) are tentative. Residue 27 was identified as lysine in the initial run.

De la Rosa *et al.*, 1981) and *Neurospora* (Le & Lederer, 1982).

There were difficulties in obtaining amino acid sequence data using undigested yeast NR, indicating that the N-terminus of the enzyme is blocked. However, the 40 kDa fragment obtained by tryptic digestion gave a single, partial sequence of 36 residues with good yield in an initial cycle (repetitive yield = 93%; initial sequence yield = 72 pmol). The sequence was extended to 50 residues in a subsequent run and for cycles 2–50 the average repetitive yield was 92% and the initial yield was 168 pmol. There was excellent agreement between both runs and no other N-terminal sequences were detected. The sequence obtained is shown in Fig. 2. The estimated isoelectric point of this sequence is 4.72. Comparison of

this sequence with the deduced amino acid sequences of higher plant and fungal NRs and other proteins stored in the National Research Foundation Protein Library or the GenBank Genetic Sequence Databank, failed to reveal significant similarity.

Effect of ionic strength, phosphate and protein on yeast NR activity

Kay & Barber (1986) have shown that increased ionic strength *in vitro* has a stimulatory effect on the activity of *Chlorella* NR. We found this also with yeast NR, but only when our assay mixtures did not contain added BSA. At both low ($I = 20$ mM) and high ($I = 100$ mM) ionic strength, the pH optimum of NADH-NR activity was 7.0.

Fig. 3 shows the effect of the presence of BSA in a similar experiment with all assays done at pH 7.0. NAD(P)H-NR activity was stimulated by 60% after an increase in ionic strength from 15 to 100 mM in assay mixtures which did not include BSA. When BSA was present, there was no such stimulation of NR activity. Further experiments showed that the ionic enhancement effect exhibited in the absence of BSA was only apparent with the terminal nitrate-reducing partial activity (BV-NR) of the enzyme (Fig. 4). Increase in ionic strength had little effect on FAD-dependent diaphorase activity (NADH-DCPIP NR).

In our *in vitro* NR assays with crude extracts and purified fractions, we routinely include BSA (Boehringer Mannheim, protein content > 98%) in an assay mixture containing approximately 25 mM-phosphate buffer (pH 7.5) at final volume, since this enhances enzyme

Fig. 3

Fig. 3. Effect of ionic strength on yeast NR activity. NR (0.625 pmol) was assayed at different ionic strengths with BSA present (○) or absent (●) by following the nitrate-dependent oxidation of NADH.

Fig. 4

Fig. 4. Effect of ionic strength on partial activities of yeast NR. NR (0.625 pmol) was assayed at different ionic strengths in the absence of BSA. ●, BV-NR activity; ○, DCPIP-NR activity.

Table 3. Effect of BSA and casein on NR activities

100 % NR activity of the enzyme preparation was equivalent to an NADH-NR activity of $324 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (a) and $300 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (b).

Addition (μg per assay)	NR activity (%)		
	NADH-DCPIP NR	BV-NR	NADH-NR
(a) BSA			
0	100	8	40
5	100	22	46
10	100	60	66
50	100	92	100
100	100	100	100
250	100	95	100
(b) Casein			
0	100	12	19
5	100	24	37
10	100	49	71
50	100	84	85
100	100	90	92
250	100	100	100

activity *in vitro* (Hipkin *et al.*, 1986). When BSA was omitted, NR activity was 40 % of the activity obtained in assays containing 50–2500 μg BSA (in an assay volume of 700 μl) (Table 3). NR activity decreased dramatically when the amount of BSA was decreased below 25 μg per assay. Moreover, it should be noted that similar increases in NR activity were observed irrespective of whether the BSA preparation had been subject to prior filtration through Superose 6 or not. Furthermore, similar results were obtained when casein was used in place of BSA (Table 3).

Further investigations of the effects of BSA and casein showed that additions of these proteins had a differential effect on the partial activities of the enzyme. As with the increased ionic strength effect, BV-NR activity was stimulated by the addition of extra protein but there was no effect on NADH-DCPIP NR activity (Table 3).

The inclusion of exogenous proteins like BSA in NR extraction and assay buffers in order to enhance activity in crude preparations is not uncommon (Schrader *et al.*, 1974; Purvis *et al.*, 1976), although their role in any activation process that may occur has not been clear. It has been suggested that BSA may protect NR from dissociation into subunits (Purvis *et al.*, 1976). Consequently, since yeast NR exists as a dimer or tetramer *in vitro*, depending on NR concentration (Hipkin *et al.*, 1986), we tested whether the protein effect described here could have resulted from a change in the quaternary configuration of yeast NR from the dimeric to the tetrameric form at high protein (NR+BSA) concentrations. However, gel-filtration of yeast NR by FPLC using Superose 6 yielded values for the Stokes'

radius of 6.3 nm, irrespective of whether the enzyme was chromatographed in the presence or absence of BSA. This indicated that BSA was neither binding to NR nor effecting a change in the quaternary structure of the enzyme, and that the dimer was the predominant form under both conditions.

Since the effects of increased protein and ionic strength were very similar and not additive at optimal concentrations of protein, we suggest that both are types of ionic effects which influence the transfer of electrons from the haem centre to the molybdenum centre of the enzyme, a rate-limiting step in the catalytic turnover of this enzyme (Kay *et al.*, 1990). Therefore, it should be noted that experiments on the effects of increased ionic strength on NAD(P)H-CR activity must be treated with caution, since assay of that partial activity involves supplying a protein (cytochrome *c*) as a substrate, which itself may contribute significantly to an ionic effect. This might explain why Barber & Notton (1990) found that maximum NADH-CR activity in spinach (*Spinacea oleracea*) was apparently achieved at a lower ionic strength than maximum activity of NADH-DCPIP NR.

Stimulation of NR activity by inclusion of phosphate in assay mixtures has been noted with fungal and algal extracts (Nicholas & Scawin, 1956; Everest *et al.*, 1984). Therefore, in order to investigate the relationship between phosphate activation and extraneous protein/ionic effects, the effect of phosphate on yeast NADH-NR activity was also studied. Initial experiments confirmed that phosphate additions to low ionic buffers resulted in activation of NR activity (not shown). Subsequently, high ionic strength assay buffer (100 mM) at pH 7.0 was used and phosphate concentrations were varied from 0–200 mM. Addition of 50–100 mM phosphate resulted in an approximately 3-fold increase in NR activity. Since the phosphate effect occurs in a high ionic environment and is both larger and more striking than any ionic strength effect we have observed, we concluded that it results from a separate type of enzyme interaction.

It is clear from recent investigations with *Chlorella* and higher plants that NR proteins from diverse eukaryotic sources show many similarities at the biochemical level (Solomonson & Barber, 1990). However, despite the rapid advances being made at the molecular-genetic level with some fungal systems, the nonavailability of stable and pure fungal NR has limited the progress of comparative studies at the biochemical level. However, our studies with the yeast *Candida nitratophila* indicate that fungal NR also shows a large number of similarities with other NR proteins at the biochemical level. We have shown here that yeast NR contains one molecule of haem and one atom of molybdenum per molecule of subunit and that the haem is associated with a 40 kDa, C-terminal, tryptic fragment. In addition, as with plant

NR (Kay & Barber, 1986; Barber & Notton, 1990), yeast NR activity is subject to stimulation by increased ionic strength and phosphate.

We have commented previously on the close similarity between yeast NR and *Chlorella* NR in terms of quaternary structure, thermodynamic properties and the rate-limitation in catalysis imposed by the intramolecular transfer of electrons between the haem and molybdenum centre (Hipkin, 1989; Hipkin *et al.*, 1989; Kay *et al.*, 1990). However, similarities are not apparent when the short sequence of amino acids for the 40 kDa tryptic fragment of yeast NR, discussed here, is compared with the deduced amino acid sequences of other (plant and fungal) NR proteins. A possible explanation for this is that the yeast NR sequence described here is from a trypsin-sensitive hinge region in the C-terminal half of the enzyme – a region where little sequence similarity is apparent when other eukaryotic NR proteins are compared with each other (Campbell & Kinghorn, 1990).

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