Structure and Properties of Assimilatory Nitrate Reductase from the Yeast *Candida nitratophila*

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NAD(P)H nitrate reductase (EC 1.6.6.2) from the yeast *Candida nitratophila* has been purified to electrophoretic homogeneity. The concentrated enzyme has a molecular mass of 365 kDa and consists of four subunits each of 95 kDa, but appears to dissociate into dimers after dilution. The absorption spectrum of the homogeneous protein is typical of a *b*-type cytochrome and its isoelectric point is pH 5.4. The enzyme utilizes both NADH and NADPH but is more active with NADH. Preincubation of pure enzyme with NAD(P)H and cyanide, but not NAD(P)H and ADP, leads to a reversible redox inactivation of the enzyme. Preincubation with NAD(P)H alone activates the enzyme.

INTRODUCTION

Assimilatory nitrate reductase (NR) has been highly purified from a number of eukaryotic sources including fungi (Guerrero & Gutierrez, 1977; Pan & Nason, 1978; Renosto *et al.*, 1981), algae (Solomonson, 1975; Giri & Ramadoss, 1979; De la Rosa *et al.*, 1980) and higher plants (Fido & Notton, 1984; Redinbaugh & Campbell, 1985). In these organisms NR is a multimeric, molybdoflavoprotein, which may differ in subunit mass and composition depending on the source from which it is obtained. Further, in algae and higher plants the preferred electron donor is often NADH whereas in fungi, it is usually NADPH (Pateman & Kinghorn, 1976; Garrett & Amy, 1978; Hipkin *et al.*, 1979; Beevers & Hageman, 1980). Some differences between eukaryotic NR proteins could be of phylogenetic importance and may be related to their mode of regulation *in vivo*.

During studies of the regulation of nitrate assimilation in yeast we observed that the NR of *Candida* spp. (*C. nitratophila*, in particular) was very active. We have purified the enzyme from *C. nitratophila* to a very high specific activity and describe here some aspects of its quaternary structure and biochemical properties.

METHODS

Organism and culture. *Candida nitratophila* (NCYC 556) was obtained from the National Yeast Culture Collection, Norwich, UK. It was grown in 250 ml Erlenmeyer flasks containing 100 ml of the liquid mineral medium described by Ferguson & Sims (1971), with 1% (w/v) glucose as carbon source and either 10 mM-NaNO₃ (nitrate medium) or 10 mM-NH₄Cl (ammonium medium) as nitrogen source. The pH of the medium after autoclaving was 5.1. Twenty 100 ml cultures were started by inoculation from stock cultures (maintained on Oxoid malt extract agar plates) into ammonium medium and these were incubated for 16 h at 28 °C with orbital shaking at 110 r.p.m. Cultures were then harvested by centrifugation at 500 *g*, washed with distilled water and resuspended in equal portions into five 180 ml portions of nitrate medium. These nitrate-adapting cultures were then incubated under growth conditions for a further 2 h.

Purification of nitrate reductase (NR) (EC 1.6.6.2). Nitrate-adapted cultures (approximately 20 g wet weight) were harvested, washed with distilled water and resuspended in 60 ml 50 mM-potassium phosphate buffer, pH 7.5,

Abbreviations: BVH, reduced benzyl viologen; pCMB, *p*-chloromercuribenzoate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NR, nitrate reductase.

containing 5 μM -FAD, 150 μM -dithiothreitol and 120 μM - Na_2EDTA (buffer A). Cell-free extract was prepared by passing this suspension two or three times through a chilled French pressure cell at 100 MPa. The homogenate was then centrifuged at 40000 g for 25 min at 3 °C and the supernatant was filtered through glass fibre paper. Subsequent purification of the filtered extract involved at least four sequential affinity chromatography runs using blue Sepharose CL-6B (Pharmacia) and elution with reduced pyridine nucleotides. The filtered extract was loaded (0.4 ml min⁻¹) onto a K9/15 column (Pharmacia) containing the blue Sepharose gel equilibrated with buffer A. NR in the crude extract does not bind to the affinity gel at this stage but elutes straight through the column in buffer A. Residual NR in the void volume was eluted with 20 ml buffer A. Active fractions were pooled and the procedure was repeated using a fresh column of blue Sepharose until NR bound to the column and could not be detected in the eluate. The column was then washed with 100 ml buffer A and NR was eluted with 30 ml buffer A containing 0.1 mM-NADH. Active fractions were pooled, left for 16 h at 4 °C and re-loaded onto another fresh column of blue Sepharose, which was washed and eluted with NADH buffer as before. Active fractions were pooled and concentrated by ultrafiltration at 400 kPa through an Amicon PM 30 filter to a volume of 1–2 ml. This concentrate was diluted to 10 ml with buffer A, concentrated again, diluted to 10 ml and re-loaded onto a fresh blue Sepharose column. This was washed with buffer A as before and NR was eluted with buffer A containing 0.1 mM-NADPH. Active fractions were pooled and concentrated as before. With some preparations the highly purified enzyme from this step was chromatographed through a 90 cm Bio-Gel 1M (Bio-Rad) column at a rate of 18 ml h⁻¹. Active fractions were pooled and concentrated as before. This final gel filtration step did not increase the degree of purification obtained but removed minor protein bands sometimes observed on electrophoresis gels.

Enzyme assays. Enzyme assay mixtures contained 50–100 μg protein from crude extracts or 0.25–0.5 μg protein from purified preparations. NAD(P)H-NR activity was assayed by nitrite production in a volume of 0.7 ml containing 12.5 μmol potassium phosphate buffer, pH 7.5, 2.5 nmol FAD, 25 μmol KNO_3 , 2.5 mg bovine serum albumin and 0.8 μmol NADH or NADPH. The assay reaction proceeded for 10 min at 30 °C and was terminated by boiling. Tubes were cooled, and, after the addition of 1 ml 30 μM -phenazine methosulphate (Scholl *et al.*, 1974), placed in the dark for 10 min. Nitrite was determined colorimetrically by the Griess–Ilosvay reaction as described by Everest *et al.* (1984). Terminal-NR (BVH-NR) activity was assayed as described above except that assay mixtures contained 0.5 μmol benzyl viologen (BV) reduced by the addition of 1.6 mg sodium dithionite and 1.6 mg NaHCO_3 . Assays were terminated by vigorous mixing, which rapidly oxidized residual dithionite.

NAD(P)H-cytochrome *c* reductase (diaphorase) activity was determined spectrophotometrically at 550 nm. Reaction mixtures (2.8 ml) contained 140 μmol potassium phosphate buffer, pH 7.5, 14 nmol FAD, 0.5 μmol NADH or NADPH, 2.5 mg bovine serum albumin and 8 mg cytochrome *c* (Sigma, horse heart). Reactions were initiated by the addition of enzyme and activity was calculated from initial reaction rates over a 60 s period. Affinity constants for the enzyme were calculated from least squares regression analysis of Hanes–Wolfe plots (Bliss & James, 1966).

Protein determinations. Protein was measured by the method of Bradford (1976), using Bio-Rad protein reagent.

Determination of the molecular mass of NR. This was done by the method of Siegel & Monty (1966). Values for Stoke's radius (r_s) were determined by gel filtration through Sephacryl S-400, superfine (Pharmacia) and Bio-Gel A-1.5 M (Bio-Rad) in 90 cm columns, as described by Hipkin *et al.* (1985). Values for sedimentation coefficient ($S_{20,w}$) were determined by sucrose density gradient centrifugation by a method based on that of Martin & Ames (1961), as described by Hipkin *et al.* (1985). The molecular mass of NR was estimated by computing values of r_s and $S_{20,w}$ into the Svedberg equation (Siegel & Monty, 1966).

Electrophoresis. SDS-PAGE was done on 10% and 12% (w/v) vertical slab gels (Laemmli, 1970). Non-dissociating electrophoresis was done at 20 mA for 3–4 h at 4 °C in 7% (w/v) polyacrylamide gel. NR activity in these gels was measured in 5 mm slices. Isoelectric focusing was done using an LKB multiphore system and LKB ampholine polyacrylamide plates with a pH gradient of 3.5–9.5 at 4 °C, according to the manufacturer's instructions. The cathodic electrode strip was saturated with 1 M-NaOH and the anodic strip with 1 M- H_3PO_4 . Electrophoresis was allowed to proceed at 1600 V for 90 min. NR activity in these gels was measured in 3 mm slices.

Protein bands on electrophoresis gels were detected by staining with Coomassie brilliant blue R.

Reproducibility of results. All experiments were repeated at least twice and representative results are presented.

RESULTS AND DISCUSSION

Purification and physical properties of NR from C. nitratophila

NR was purified after four or five sequential chromatography steps using the affinity gel blue Sepharose CL-6B. During the first run 90% of the NR activity loaded onto the column eluted without binding. Other proteins present in the crude extract, however, did bind to the gel. During subsequent steps, NR bound to the gel and could be eluted with 0.1 mM-NADH. A final

Table 1. Purification of *C. nitratophila* NR

	Vol. (ml)	Total protein (mg)	Activity ($\mu\text{mol min}^{-1}$)	Yield (%)	Specific activity ($\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$)	Purification (fold)
Crude extract	60	727	144	100	0.198	0
Blue Sepharose CL-6B affinity chromatography						
Run 1 (no binding)	75	397	135	95	0.34	2
Run 2 (NADH elution)	30	7.28	105	73	14.42	73
Run 3 (NADH elution)	10	1.29	75	52	58.23	294
Run 4 (NADPH elution)	10	0.276	60	42	217.40	1098

affinity run using 0.1 mM-NADPH to elute the enzyme resulted in a preparation that had been purified by at least 1000-fold with a specific activity of not less than 200 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ [3.3 μkat (mg protein) $^{-1}$]. Yields varied between 20 and 50% with recoveries of 150–300 μg NR protein. Sixteen separate batches of *C. nitratophila* extracts were purified by this procedure with similar results. The results of a typical run are shown in Table 1.

Purified NR was not stable during storage in buffer A at 4 °C, 50% of the enzyme activity being usually lost after 7 d, and was particularly labile to dilution with protein-free buffer. For this reason 2.5 mg bovine serum albumin was included in all enzyme assays to stabilize the enzyme.

The purity and subunit composition of the enzyme were estimated by electrophoresis. Only one protein band was evident on non-dissociating gels and its position coincided with a sharp peak in BVH-NR activity measured in gel slices (Fig. 1). Also, on SDS gels freshly purified enzyme showed one band with a molecular mass estimated as 95 ± 1.0 kDa (Fig. 2).

The r_s of the freshly purified enzyme was estimated to be 8.5 nm. Older, less active preparations of pure NR gave a lower r_s (5.4 nm). Values for the $S_{20,w}$ of the enzyme were dependent upon the concentration of NR applied to the top of the sucrose gradient; with 200 μg protein ml^{-1} a value of 10.25 was obtained consistently, decreasing to 8.75 (or 7.8 in one experiment) with 50 μg protein ml^{-1} . The dilute enzyme also showed a lower r_s (6.5 nm). When the values $r_s = 8.5$ nm and $S_{20,w} = 10.25$ (i.e. concentrated, freshly purified enzyme) are computed with the Svedberg equation, a molecular mass of 365 kDa is obtained. Given a subunit mass of 95 kDa, this species of the NR molecule appears to be composed of four subunits. A molecular mass of about 200 kDa was calculated for the dilute enzyme, which appears to be composed of two subunits. The isoelectric point of the enzyme was pH 5.4. Enzyme activity in gel slices coincided with a major band although the presence of minor, non-active bands suggested that the enzyme dissociated as it migrated towards the anode (not shown).

The difference spectrum (reduced minus oxidized enzyme) of the pure enzyme indicated absorption maxima for the reduced enzyme at 423 nm (Soret band), 557 nm and a broad shoulder centred around 528 nm. The oxidized enzyme showed an oxidized Soret peak at 413 nm (Fig. 3). From this we infer the presence of cytochrome b_{557} in the enzyme molecule. The A_{278}/A_{413} ratio of the enzyme used in this experiment was 2.4.

Estimations of the molecular mass of eukaryotic NR vary from approximately 200 kDa to 500 kDa. Of the more highly purified and well studied enzymes, that of *Neurospora crassa* appears to have a molecular mass of 290 kDa with two subunits (Horner, 1983), whereas that of *Chlorella* appears to have a molecular mass of 360 kDa made up of four subunits (Howard & Solomonson, 1982). The quaternary structure of *C. nitratophila* NR, described here, closely resembles that of *Chlorella* NR (Howard & Solomonson, 1982). Moreover, the latter showed that the *Chlorella* enzyme can exist in various active states of polymerization *in vitro*, depending on dilution, and they suggested that the active protein may exist as a dimer or a dimer of dimers.

Fig. 1

Fig. 1. Non-dissociating PAGE of purified *C. nitratophila* NR. Samples containing approx. 40 μ g pure NR were loaded onto a 7% (w/v) polyacrylamide gel and electrophoresed for 3–4 h at 4 °C. The gel was then divided and half was stained for protein (a). The other half was cut into 5 mm slices, which were then assayed for BVH-NR activity (b).

Fig. 2

Fig. 2. SDS-PAGE of purified *C. nitratophila* NR. Purified NR was electrophoresed on a 12% (w/v) SDS-polyacrylamide gel and stained for protein. Track 1 contains marker proteins and tracks 2 and 3 were loaded with 25 μ g and 50 μ g of pure NR respectively.

Fig. 3. Absorption spectra of pure *C. nitratophila* NR measured on a Pye-Unicam SP 800 twin beam scanning spectrophotometer. NR was reduced by the addition of a few crystals of sodium dithionite.

Table 2. Pyridine nucleotide specificity and flavin requirement of *C. nitratophila* NR

Addition*	Percentage NR activity†
NADH	40.4
NADH + FMN	73.4
NADH + FAD	100.0
NADPH	18.3
NADPH + FMN	28.4
NADPH + FAD	59.6

* NAD(P)H, 240 μ M; FMN, 5 μ M; FAD, 5 μ M.

† 100% NR activity = 197.8 μ mol NO₂⁻ min⁻¹ (mg protein)⁻¹.

Table 3. Activation and inactivation of *C. nitratophila* NADH-NR activity

Preincubation addition*	Percentage activity†	Preincubation addition*	Percentage activity†
None	100.0	KCN	98.0
NADH	140.5	NADH + KCN	12.3
NADPH	115.8	NADH + KCN (+ferricyanide)	132.3
ADP	98.7	KNO ₃ + NADH + KCN	118.2
NADH + ADP	145.6	pCMB	14.3
NADH + ADP + Mg ²⁺	139.3	NADH + pCMB	123.3
		NADPH + pCMB	115.8

* Purified NR was preincubated with NAD(P)H or ADP for 60 min at 0 °C, or with KCN or pCMB for 10 min at 20 °C. NAD(P)H, 0.3 mM; ADP, 0.3 mM; MgCl₂, 10 mM; KCN, 3 μ M; ferricyanide, 0.3 mM; KNO₃, 5 mM. pCMB, 20 μ M.

† 100% NADH-NR activity = 282.1 μ mol NO₂⁻ min⁻¹ (mg protein)⁻¹.

Our results suggest a similar variation in quaternary structure, based on dimers, for *C. nitratophila* NR. Losses of activity that occur after dilution or storage may result from dissociation of the tetrameric form of the enzyme. The freshly purified and concentrated protein, stabilized with bovine serum albumin, had a very high specific activity and a turnover number of >1000 s⁻¹.

Pyridine nucleotide specificity and in vitro inactivation/activation of NR

Unlike the NR of other fungi such as *Aspergillus*, *Neurospora*, *Penicillium* and *Sporobolomyces*, which are NADPH-specific (Cove & Coddington, 1965; Garrett & Amy, 1978; Renosto *et al.*, 1981; Ali & Hipkin, 1985), the NR of *C. nitratophila* could utilize NADH and NADPH (Table 2). However, NR activity was 50% greater with NADH. Maximum activity required the addition of flavin nucleotide, and FAD was more effective than FMN. Apparent K_m values for pyridine nucleotides were 16.9 $\pm$ 5.1 μ M (NADH) and 29.8 $\pm$ 9.7 μ M (NADPH), and those for nitrate were 120.3 $\pm$ 30.4 μ M (NADH-NR) and 110.0 $\pm$ 20.7 μ M (NADPH-NR).

Like the NR of *Neurospora* and unicellular algae (Garrett & Greenbaum, 1973; Solomonson *et al.*, 1973; Hipkin *et al.*, 1979), *C. nitratophila* NR was subject to a reversible, redox inactivation in the presence of NADH and cyanide. Preincubation with cyanide and NADH inactivated enzyme activity by 88% and the inactive enzyme was reactivated after treatment with ferricyanide. Addition of cyanide alone did not inactivate the enzyme (Table 3). Addition of nitrate to preincubation mixtures before cyanide and NADH protected the enzyme against inactivation. NR from certain algal sources is subject to a reversible redox inactivation after long term incubation with NAD(P)H or, particularly, with NAD(P)H and ADP (Moreno *et al.*, 1972; Maldonado *et al.*, 1973). The NR of *C. nitratophila* was not inactivated by such treatment (Table 3); in contrast, preincubation with NADH or NADPH activated NR activity as long as cyanide was absent, the presence or absence of ADP having little effect. Preincubation of the enzyme with pCMB inhibited NR activity by 86% (Table 3), which was prevented when

Table 4. *Activation and inactivation of terminal-NR (BVH-NR) and NADH-cytochrome c reductase*

Addition*	Percentage BVH-NR activity†	Percentage NADH-cytochrome c reductase activity‡
None	100.0	100.0
NADH	129.6	140.6
NADH + KCN	5.5	163.4
pCMB	102.9	2.3

* NADH, 0.3 mM; KCN, 3 μ M; pCMB, 20 μ M.

† 100% BVH-NR activity = 157.6 μ mol NO₂⁻ min⁻¹ (mg protein)⁻¹.

‡ 100% NADH-cytochrome c reductase activity = 382.8 μ mol min⁻¹ (mg protein)⁻¹.

NADH or NADPH was added before pCMB. Inhibition by cyanide and NADH was the result of a specific inactivation of BVH-NR activity, whereas pCMB inactivation only inhibited cytochrome c reductase activity (Table 4). The addition of low concentrations of pCMB (4–16 μ M) enhanced BVH-NR activity (not shown). Preincubation with NADH, which activated NR activity, enhanced both BVH-NR and cytochrome c reductase activities (Table 4). We do not understand the mechanism of this phenomenon but it is possible that NAD(P)H stabilizes the protein in a more active, catalytic configuration. It is noteworthy that NADPH prevented the decay of NR activity in *Aspergillus nidulans* extracts (Dunn-Coleman & Pateman, 1976).

Our principal aim in purifying and characterizing the NR of *C. nitratophila* has been to compare the structure and properties of a yeast NR with that of eukaryotic NR from other sources. Our purified preparations had a high specific activity and a high catalytic activity, which compares with the most highly purified preparations of eukaryotic NR (Pan & Nason, 1978; Renosto *et al.*, 1981). The yeast enzyme resembles *Chlorella* NR in many respects but differs in its ability to use both NADH and NADPH as electron donors. We made use of this property when we developed our procedure for purifying *C. nitratophila* NR to homogeneity. Our recent experiments (unpublished results) indicate that several other yeast species possess bispecific NR and that the elution procedure described here may be used in their purification.

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