On the presence of a heme-binding domain homologous to cytochrome b_5 in *Neurospora crassa* assimilatory nitrate reductase

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Assimilatory nitrate reductase has been purified with 55% recovery from a *Neurospora crassa* nmr-1 nit-6 mutant, using a modification of a published procedure. It possesses one heme per 240 000 g, and subunits of mol. wt. 68 000. Upon digestion with chymotrypsin, a heme-binding domain was isolated by gel filtration; its visible spectrum was highly similar to that of cytochrome b_5 . On SDS gels, the fraction showed two heme-containing bands of $\sim 10\,000$ and $12\,500$ daltons; their amino acid composition was not very different, suggesting that they originated from the same region of the polypeptide chain. After S-carboxymethylation, the mixture of bands was submitted to cyanogen bromide cleavage, and the fragments were separated by h.p.l.c. The two largest fragments yielded an identical sequence upon automated degradation. This sequence (39 residues with some gaps) could be easily aligned with that of cytochrome b_5 starting close to the N terminus. These results are discussed in terms of the possible quaternary structure of *N. crassa* nitrate reductase, whose heme-binding domain proves to be another member of the family of b_5 -like cytochromes.

Key words: cytochrome b_5 /evolution/*Neurospora crassa*/nitrate reductase/structural domain

Introduction

Pyridine nucleotide-dependent assimilatory nitrate reductase controls what is considered to be the rate-limiting step in nitrate reduction to ammonia. It is found in fungi, green algae and higher plants. It has been purified from a number of sources and is known to contain three cofactors: FAD, protoheme IX and molybdenum. Structural characterisation in terms of the stoichiometry of prosthetic groups and subunits has shown a diversity of quaternary structures (Hewitt, 1975; Garrett and Klein Amy, 1978; Guerrero *et al.*, 1981).

Detailed structural investigation has proven difficult due to low abundance of the enzyme and to its frequently noted instability when pure. The architecture of this protein, however, should be of interest. Insofar as it possesses three prosthetic groups, one would expect it to be composed of structural domains. Analogous cases which have been well documented are those of the two bifunctional redox enzymes, the flavohemoprotein flavocytochrome b_2 and the molybdohemoprotein sulfite oxidase. They have been shown to consist of the juxtaposition of a heme-binding domain and either a flavodehydrogenase domain (Labeyrie *et al.*, 1966; Gervais and Tegoni, 1980), or a molybdoprotein domain (Ito, 1971; Guiard and Lederer, 1977; Johnson and Rajagopalan, 1977), respectively. In both cases the heme-binding domain could be isolated after selective proteolysis. Its visible spectrum was

highly similar to that of microsomal cytochrome b_5 , with which sequence data showed a common evolutionary origin (Guiard and Lederer, 1979a, 1979b; Guiard *et al.*, 1974).

When it was first described, the chromophore of nitrate reductase was called cytochrome b_{557} , and its spectral similarity with microsomal cytochrome b_5 and baker's yeast L-lactate dehydrogenase (flavocytochrome b_2) was stressed (Garrett and Nason, 1969b). The question therefore arises as to whether nitrate reductase also contains a heme-binding domain homologous to cytochrome b_5 . Here we report the results of proteolysis experiments carried out with purified nitrate reductase from *Neurospora crassa*. They prove that this enzyme has a protease-stable heme-binding domain made of a continuous stretch of peptide chain of ~ 100 residues which presents the native cytochrome b_5 -like spectrum. Partial sequence data indicate homology with cytochrome b_5 . Furthermore, we report analytical data concerning the heme stoichiometry and subunit mol. wt. in the native enzyme which are at variance with some previously reported results. These data have some bearing on the problem of the protein quaternary structure.

Results

Nitrate reductase purification and characterisation

The final steps of the purification scheme described in Materials and methods were modified from that devised by Pan and Nason (1978). Some steps were omitted but another was added, namely chromatography on blue dextran-Sepharose (Solomonson, 1975) from which the enzyme could be eluted by NADPH at pH 6 as described in Materials and methods, or at pH 7 by $100\,\mu\text{M}$ NADPH and $50\,\mu\text{M}$ FAD. We obtained a final recovery of 50–55%, compared with 30% for the published procedure (Table I). The recovery was also 50% with a wild-type strain, but in that case the final amount was reduced by 5- to 6-fold compared with that given in Table I.

Under non-denaturing conditions, the electrophoresed preparation showed a single band which could be stained for protein (Figure 1B), heme and reduced methyl viologen-nitrate reductase activity (not shown). Under denaturing conditions, a single band was also observed which could be stained for both protein (Figure 1A) and heme (not shown). Its apparent mol. wt. was 68 000, slightly higher than that of the bovine serum albumin marker.

The amino acid composition of the protein is given in Table II. It shows a general similarity to those of nitrate reductase from *Chlorella vulgaris* (Giri and Ramadoss, 1979), *Ankistrodesmus braunii* (De la Rosa *et al.*, 1981) and barley (*Hordeum vulgare*) (Kuo *et al.*, 1982). The spectrum of the enzyme is presented in Figure 2A; its characteristic absorbance ratios are given in Table III. Using $\epsilon_{\text{red}}^{423} = 180\,\text{mM}^{-1}\,\text{cm}^{-1}$, a correlation between heme content and protein weight determined by amino acid analysis yielded a figure of $1\,\mu\text{mol}$ heme per $240 \pm 24\,\text{mg}$ (average of six preparations).

Chymotryptic proteolysis of nitrate reductase

Several proteases were used in our attempts to detect the presence of a low mol. wt. heme-binding domain in nitrate

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Table I. Nitrate reductase purification

Purification step	Total volume (ml)	Total protein ^a (mg)	Total activity (units × 10 ⁻³)	Specific activity (units/mg)	Recovery (%)
Homogenate	3000	29 000	1800	61	100
(NH ₄) ₂ SO ₄ precipitation	300	18 000	1548	86	86
DEAE Sephadex	200	9800	1416	144	79
FAD Sepharose	15	68	1137	16 720	63
Blue-dextran-Sepharose	16	32	1006	31 450	56

^aAssayed by the Lowry procedure (Lowry *et al.*, 1951).

The data refer to the extraction of 1 kg (fresh weight) of the *N. crassa* nmr-1 nit-6 mutant.

Fig. 1. Gel electrophoresis of nitrate reductase. **A**, SDS gel electrophoresis (7.5% polyacrylamide) (Weber and Osborn, 1969). **a**: purified nitrate reductase (the lane on the left was loaded with three times as much material as the lane in the center); **b**: fraction eluted from DEAE-Sephadex; **c**: fraction eluted from FAD-AH-Sepharose; **d**: ovalbumin; **e**: bovine serum albumin; **f**: human transferrin; **g**: β -galactosidase. **B**, Gel electrophoresis under non-denaturing conditions (7.5% polyacrylamide) (Davis, 1964).

reductase: trypsin, *Staphylococcus aureus* V8 protease and chymotrypsin. The protein appeared fairly resistant to trypsin but could be digested with the other two proteases. The experiments with chymotrypsin, which gave the most satisfactory results, are the only ones described here. Figure 3 shows the profile of a Sephadex G-50 separation of the digestion products. The last peak contained flavin, the third peak must have contained u.v.-absorbing peptides, none of which were visible on SDS acrylamide gels. The minor heme-containing peak was incompletely digested material. The major heme-containing peak showed two protein bands upon electrophoresis under non-denaturing conditions (Figure 4B) as well as denaturing ones (Figure 4A). In all cases they both stained for heme (not shown). These bands were slightly faster than tryptic beef cytochrome *b*₅ (86–93 residues) and slightly slower than horse heart cytochrome *c* (104 residues), respectively.

Table II. Amino acid composition of nitrate reductase and of its heme-binding fragment

Amino acid	Nitrate reductase ^a (mol %)	Heme-binding domain ^b (mol %)	Domain heavy band (mol %)	Domain light band (mol %)
Asp	10.25	11.00	9.71	9.46
Thr	5.98	6.31	7.58	7.29
Ser	5.90	6.96	9.30	9.15
Glu	14.36	14.21	9.10	6.59
Pro	7.84	6.31	5.85	7.13
Gly	8.62	9.33	13.37 ^c	15.50 ^c
Ala	8.15	8.54	8.96	11.32
1/2 Cys	nd	0.28	nd	nd
Val	6.75	5.81	7.17	7.21
Met	1.63	0.86	0.69	0.93
Ile	4.11	3.30	3.03	2.63
Leu	7.76	7.18	6.89	7.75
Tyr	2.95	2.51	1.58	1.16
Phe	3.26	2.94	3.86	0.85
His	3.03	2.22	1.86	1.78
Lys	5.74	5.74	5.85	4.73
Arg	3.65	2.73	1.31	2.17
Trp	nd	0.28	nd	nd

nd, not determined.

^aAverage of duplicate values obtained for seven different preparations.

^bAverage of duplicate values obtained for six different preparations, except for cysteine (two values, determined as S-carboxymethylcysteine) and tryptophan (two values).

^cThese high values most probably arise from the presence of contaminants (in particular from electrophoresis buffer components).

The absorption spectrum of the Sephadex fraction is shown in Figure 2. It is obviously highly similar both to that of the native enzyme and that of microsomal cytochrome *b*₅. Its characteristic ratios are reported in Table III. The amino acid composition of the fragment is presented in Table II. With $\epsilon_{\text{red}}^{423} = 180 \text{ mM}^{-1} \text{ cm}^{-1}$, a figure of $1 \mu\text{mol heme}/12.2 \pm 0.9 \text{ mg}$ was established by a correlation between the spectrum and the amino acid content for six different preparations of the heme-binding domain. This figure is in satisfactory agreement with the indications of SDS-gel electrophoresis and confirms the fact that both protein bands contained heme.

The amount of flavin eluted from the Sephadex column (Figure 3) was estimated by fluorimetry and correlated with the amount of eluted heme. For three different preparations the average was $0.85 \pm 0.25 \text{ mol flavin/mol heme}$. The overall recovery of heme-binding components was $\sim 80\%$.

The N-terminal amino acid of the heme-binding domain eluted from Sephadex was found to be heterogeneous. In order to find out whether the two bands seen on SDS gels corresponded to the same area of the polypeptide chain or to two

Fig. 2. Spectra of nitrate reductase and its heme-binding domain. **A**, Nitrate reductase (— oxidised enzyme; - - - dithionite-reduced enzyme). **B**, cytochrome b_5 (—) and nitrate reductase heme-binding domains (- - -) in the oxidized state. **C**, Cytochrome b_5 (—) and nitrate reductase heme-binding domain (- - -) after dithionite reduction. Since cytochrome b_5 and the heme-binding domain have the same λ_{max} for the Soret band, in b and c the cytochrome b_5 spectrum is displaced to the right for easier visualization.

Table III. Absorbance ratios of nitrate reductase and its heme-binding domain

Ratio	Native nitrate reductase	Heme-binding domain	Cytochrome b_5
$\gamma_{\text{red}}/\gamma_{\text{ox}}$ (423,413)	1.38	1.46	1.45
$\gamma_{\text{red}}/\beta_{\text{red}}$ (423,528)	1.90	1.78	1.78
$\gamma_{\text{red}}/\alpha_{\text{red}}$ (423,557)	6.90	6.80	6.57
UV/ γ_{ox} (280,413)	0.61	0.41	

The figures in parentheses refer to the respective wavelengths in nm. The published values of $\epsilon_{\text{red}}^{423}$ are $171 \text{ mM}^{-1} \text{ cm}^{-1}$ for microsomal cytochrome b_5 (Ozols and Strittmatter, 1964), $183 \text{ mM}^{-1} \text{ cm}^{-1}$ for flavocytochrome b_2 (Pajot and Groudinsky, 1970) and $149 \text{ mM}^{-1} \text{ cm}^{-1}$ for sulfite oxidase (Cohen and Fridovich, 1971).

entirely different ones, they were eluted from a preparative SDS gel and their amino acid compositions were determined (Table II). They showed an overall similarity, with marked differences for only some amino acids (in particular glutamic acid, alanine, phenylalanine and arginine). These results suggested that the heavier band possessed the same amino acid sequence as the lighter one with an additional N-terminal extension and perhaps also a C-terminal one.

As the analytical data indicated the presence of one methionine/mol heme-binding domain, the mixture was cleaved at methionine with cyanogen bromide, and fractionated by h.p.l.c. Only three peaks contained enough material for characterization, but the smallest peptide could not be degraded in the liquid phase sequencer. On the other hand, the other two presented the same N terminus. The combined results of the two degradations are given in Table IV, together with an alignment of the determined sequence with that of known members of the cytochrome b_5 family.

Fig. 3. Purification of the heme-binding domain. —, 280 nm; - - -, 413 nm. Nitrate reductase was dialysed overnight at 4°C against 0.1 M ammonium acetate pH 8 in order to eliminate PMSF. The protein (107 nmol heme) was digested with 0.5 mg chymotrypsin for 24 h at 37°C ; at that point gel electrophoresis showed most of the 68 K band to have disappeared. The sample was applied to a column (1 x 100 cm) of Sephadex G 50 equilibrated at 4°C in 0.1 M ammonium acetate. Elution was carried out at a flow rate of 12 ml/h.

Discussion

Pan and Nason (1978) found in *N. crassa* nitrate reductase 1.5 mol heme for a mol. wt. of 228 000 daltons (Garrett and Nason, 1969a). This was assumed to indicate the presence of two heme groups. On the other hand, Jacob and Orme-Johnson (1980) found only 1 heme/228 000 g. More recently,

Tachiki and Nason (1983) revised the mol. wt. to 270 000. Our value of 1 mol heme/240 000 g $\pm$ 24 000 is compatible with both published mol. wt. values but suggests that Pan and Nason (1978) overestimated the heme content. Our correlation is of course highly dependent on the value of 180 mM⁻¹ cm⁻¹ taken as heme extinction coefficient. In view of the spectral similarity between cytochrome b₅ and nitrate reductase (Figure 2 and Table III), it appears however that our figure can hardly be in error by > 10%.

Flavin determination after proteolysis yielded a stoichio-

metry of 0.85 mol flavin/mol heme, which indicates a 1/1 ratio. The enzymatic activity was raised by ~50% when 5 μ M FAD was added to the assay solution, as proposed by Garrett and Nason (1969b). But, at the low reductase concentrations used for the assays, this may have reflected reversal of flavin dissociation rather than reconstitution after previous flavin loss. Altogether, we believe it rather unlikely that there could be 2 FAD groups/heme. Let us recall that the molybdenum content is thought to be 1 mol/mol enzyme (Pan and Nason, 1978; Jacob and Orme-Johnson, 1980).

Our estimate of the subunit mol. wt. as 68 000 daltons is much lower than the figure of 130 000 and 115 000 reported by Pan and Nason (1978). More recently, Horner (1983) determined a figure of 145 000 daltons and concluded that the enzyme purified by Pan and Nason had undergone proteolysis. In our case, we cannot exclude that proteolysis took place even in the presence of EDTA and phenylmethylsulfonyl fluoride (PMSF), since we found the enzyme to be unstable when stored at 4°C. Nevertheless, we obtained identical results for a dozen preparations using two different strains. If proteolysis occurred during our purification, the process must have satisfied the following conditions: (i) provoke little if any alteration of enzymatic activity, since our final recovery was 55%; (ii) induce no alteration of active enzyme electrophoretic behaviour, since the activity stain indicated the same mobility in non-denaturing gels throughout the purification; (iii) result in unique and complete splitting, since the purified protein showed only one band in SDS gels after the blue dextran-Sepharose step (Figure 1A).

Whatever the case, the presence of only 1 mol heme for several subunits raises a structural problem: in spite of identical mol. wt., these subunits must be chemically different. If the prosthetic group content is 1 mol each/mol enzyme, as strongly suggested by our results, an alternative to be considered is the existence of only one peptide chain for the native enzyme. It would be highly susceptible to proteolysis owing to its organization into domains, a structure made likely by the multifunctional character of the enzyme. Nitrate reductases from other sources all appear to possess more than one subunit and present highly variable quaternary structures (Ahmed and Spiller, 1976; De la Rosa *et al.*, 1980; Guerrero and Gutierrez, 1977; Howard and Solomonson, 1982; Minagawa and Yoshimoto, 1982; Small and Wray, 1980; Somers *et al.*, 1982; Steiner and Dowey, 1982; Zauner and

Fig. 4. Gel electrophoresis of nitrate reductase heme-binding domain. **A**, Gradient gel electrophoresis in the presence of SDS (12.5–17.4% polyacrylamide according to Douglas *et al.*, 1978). **a**: bovine tryptic cytochrome b₅; **b**: nitrate reductase heme-binding domain; **c**: horse heart cytochrome c; **d**: bovine pancreatic ribonuclease. **B**, Gel electrophoresis under non-denaturing conditions (7.5% polyacrylamide) of nitrate reductase heme-binding domain.

Table IV. Partial amino acid sequence of nitrate reductase heme-binding domain and alignment with known members of the cytochrome b₅ family.

<i>Neurospora</i> nitrate reductase	D L E Y E I K Q Y H ? N ? K ? – T W L I L H Y K ? Y D L T K F ? ? ? H P G G ? E
	1 5 10 15 20 25 30 35
Microsomal b ₅ (rat)	Y Y T L E E I E K H K D S K S – T W V I L H H K V Y D L T K F L E E H P G G E E
Mitochondrial b ₅ (rat)	Y Y R L E E V A K R N T A E E – T W M V I H G R V Y D I T R F L S E H P G G E E
	6 10 15 20 25 30 35 40
<i>Saccharomyces</i> b ₂ core	K I S P A E V A K H N K P D D – C W V V I N G Y V Y D L T R F L P N H P G G Q D
	10 15 20 25 30 35 40 35
Chicken sulfite oxidase	R Y T R E E V G R H R S P E E R V W V T H G T D V F D V T D F V G L H P G G P D
	6 10 15 20 25 30 35 40

The nitrate reductase partial sequence given on the first line was determined with ~1.8 nmol of a cyanogen bromide peptide. The average repetitive yield was 98%. The residues were identified by h.p.l.c. of phenylthiohydantoin; for positions 1–10 the identifications were confirmed by amino acid analysis after back hydrolysis. The degradation of a second cyanogen bromide peptide gave identical results up to position 29, after which no identification was possible. The sequence numbering for both cytochromes b₅ is that adopted in Lederer *et al.* (1983). The numbering of cytochrome b₂ core sequence starts at position 1 of intact flavocytochrome b₂ (Guiard *et al.*, 1975), that of sulfite oxidase heme-binding domain is taken from Guiard and Lederer (1979a). The alignment of the latter two proteins with microsomal cytochrome b₅ has been presented before (Guiard and Lederer, 1979b).

Dellweg, 1983). Therefore, the quaternary structure of *Neurospora* nitrate reductase cannot be inferred from that of the enzyme from other organisms and the problem deserves further investigation.

The existence of domains in the enzyme from various sources has been suggested before by proteolysis studies (Small and Wray, 1980; Brown *et al.*, 1981; Yamana *et al.*, 1980; Batt and Wallace, 1983). The present work provides, to our knowledge, the first unambiguous demonstration of the existence of at least one independently structured domain, namely the heme-binding domain, which is characterized as a one chain component. Our data suggest that the two bands observed on acrylamide gels (Figure 4) cover the same stretch of polypeptide chain with different cleavage points at the two ends. The two sequenced cyanogen bromide peptides with an identical N terminus probably differed at their C terminus, since they eluted differently from μ Bondapak C18. In view of the N-terminal heterogeneity of the heme-binding domain preparation, one would have expected to isolate at least two homoserine-containing peptides corresponding to the N-terminal part of the domain. Only one was found, however, the second one was probably lost on the reverse-phase column.

Whatever the case, our sequence results clearly show an unmistakable sequence similarity with other proteins of the cytochrome b_5 family. Surprisingly enough, the presently available data indicate the similarity to be highest with microsomal cytochrome b_5 since out of 31 positions compared, 20 are identical. The number of identical residues ranges from 18 to 21 for the comparison with known microsomal cytochromes b_5 from other species, and drops to 13, 11 and 10 identities with mitochondrial cytochrome b_5 , cytochrome b_2 core and sulfite oxidase, respectively. This degree of sequence similarity is indicative of a divergent evolutionary relationship between the heme-binding domain of assimilatory nitrate reductase and those of cytochrome b_5 , flavocytochrome b_2 and sulfite oxidase.

It is known that assimilatory nitrate reductase possesses the same molybdenum cofactor as sulfite oxidase (Johnson *et al.*, 1980). Therefore, it is expected to incorporate in a single molecular edifice the molybdenum-heme functional interactions present in sulfite oxidase and the flavin-heme interactions of flavocytochrome b_2 or of the NADH cytochrome b_5 reductase-cytochrome b_5 complex. Since the three-dimensional structure of beef microsomal cytochrome b_5 is determined at 2 Å resolution (Mathews *et al.*, 1971, 1972) it can be hoped that the detailed comparisons made possible when the full sequence of nitrate reductase heme-binding domain becomes available will yield some clues as to the inter-domain contacts and the mode of intramolecular electron transfer between the prosthetic groups.

Materials and methods

Strain and culture conditions

A double mutant (nmr-1 nit-6) of *N. crassa* was used for enzyme purification; it was kindly given by Dr. R.H. Garrett (Tomsett *et al.*, 1981). According to his directions, it was maintained on Vogel's medium, 2% sucrose, 1.5% agar slants containing 20 mM glutamate. Liquid cultures were carried out in Vogel's medium complemented with 30 g/l saccharose and 10 mM sodium glutamate; ammonium nitrate was replaced by sodium nitrate. The conidia of one slant were used for inoculating 50 ml culture medium in 300 ml Erlenmeyer flasks. After 24 h shaking at 25°C, two flasks were used for inoculating 1 l culture medium in 6 l Erlenmeyer flasks. After 30 h shaking at 25°C, 10 such flasks were used for inoculating a 200 l fermentor. After 48 h under aeration at 25°C, sufficient sodium nitrate was added to give a concentration

of 20 mM. After a further 4 h, the culture was harvested. The yield was 3 kg fresh weight.

Acrylamide gel electrophoresis

Gel electrophoreses were carried out in a vertical flat bed apparatus, in the presence of 0.1% SDS, using either a 7.5% polyacrylamide gel (Weber and Osborn, 1969) or an exponential gradient from 12.5 to 17.4% (Douglas *et al.*, 1978). For benzidine staining, SDS was replaced by lithium dodecylsulfate in all electrophoresis buffers as described by Delepelaire and Chua (1979). Electrophoresis under non-denaturing conditions was carried out in 7.5% slab gels according to Davis (1964). For protein elution, the gel was crushed and incubated overnight in electrophoresis buffer; the fragments were then sedimented by centrifugation and washed once with buffer. The supernatants were combined, dialysed against 20% acetic acid and lyophilised. The same gels were stained for heme with benzidine (Delepelaire and Chua, 1979) and subsequently for protein with Coomassie blue (Douglas *et al.*, 1978). Active enzyme bands were located on non-denaturing gels as described by Garrett and Nason (1969a). The gel was incubated for 4 min in 0.1 M phosphate buffer pH 7.3, 2.5 mM methylviologen and 0.08% sodium dithionite; the gel was then transferred to a 0.1 M NaNO₃ solution, and enzyme bands appeared as transient white streaks on a blue background. The gel was stained for protein after this treatment.

Protein chemistry

The protein was reduced and carboxymethylated according to Crestfield *et al.* (1963). Protein hydrolysis, amino acid analyses and automated degradations were carried out as described in Lederer *et al.* (1983). Cleavage at methionine of ~25 nmol S-carboxymethylated heme-binding domain was carried out in 260 μ l 70% formic acid with 0.26 mg cyanogen bromide for 48 h at room temperature. The peptides were separated on a μ Bondapak C18 column with a 2 h linear gradient between 100% buffer A (0.05 M aqueous trifluoroacetic acid) and 100% buffer B (0.04% trifluoroacetic acid in 50% aqueous acetonitrile) at a flow rate of 1 ml/min.

Enzyme purification

All operations were carried out at 4°C. All buffers contained 0.5 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM PMSF. Frozen mycelium (1 kg fresh weight) was homogenized in a Waring blender in 1 l 20mM phosphate buffer pH 7.3 in the presence of 500 g glass beads. After centrifugation at 10 000 g for 15 min, the residue was extracted twice more with 1 l buffer. Streptomycin sulfate precipitation, ammonium sulfate precipitation and DEAE-Sephadex A-50 chromatography were carried out essentially as described by Pan and Nason (1978). The protein was then immediately submitted to the FAD-AH-Sepharose affinity step described by Pan and Nason (1978), omitting their Biogel chromatography and isoelectric-focusing steps. After the affinity column, active fractions were concentrated by ammonium sulfate precipitation and dialysed against 10 mM phosphate buffer pH 6. The sample was applied onto a column (1.5 x 15 cm) of blue-dextran Sepharose (Ryan and Vestling, 1974) equilibrated with the same buffer. After adsorption, the column was washed with 30 ml of the buffer, then 30 ml 0.3 M phosphate buffer pH 6. After re-equilibration in the starting buffer, the protein was eluted by adding 0.1 mM NADPH to the eluent.

Enzyme assay

NADPH nitrate reductase activity was determined at 30°C in 0.1 M phosphate buffer, 5 μ M FAD, 100 μ M NADPH, pH 7.3. The specific activity was expressed as nmol NADPH oxidized/min/mg protein.

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