Biochimica et Biophysica Acta, 523 (1978) 297—313

BBA 68414

PURIFICATION AND CHARACTERIZATION OF HOMOGENEOUS ASSIMILATORY REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-NITRATE REDUCTASE FROM *NEUROSPORA CRASSA*

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(Received September 5th, 1977)

Summary

Neurospora crassa wild type STA4 NADPH-nitrate reductase (NADPH : nitrate oxidoreductase, EC 1.6.6.3) has been purified 5000-fold with an overall yield of 25—50%. The final purified enzyme contained 4 associated enzymatic activities: NADPH-nitrate reductase, $FADH_2$-nitrate reductase, reduced methyl viologen-nitrate reductase and NADPH-cytochrome *c* reductase.

Polyacrylamide gel electrophoresis yielded 1 major and 1 minor protein band and both bands exhibited NADPH-nitrate and reduced methyl viologen-nitrate reductase activities. SDS gel electrophoresis yielded 2 protein bands corresponding to molecular weights of 115 000 and 130 000. A single *N*-terminal amino acid (glutamic acid) was found and proteolytic mapping for the two separated subunits appeared similar. Purified NADPH-nitrate reductase contained 1 mol of molybdenum and 2 mol of cytochrome b_{557} per mol protein. Non-heme iron, zinc and copper were not detectable. It is proposed that the *Neurospora* assimilatory NADPH-nitrate reductase consists of 2 similar cytochrome b_{557}-containing 4.5-S subunits linked together by one molybdenum cofactor. A revised electron flow scheme is presented.

p-Hydroxymercuribenzoate inhibition was reversed by sulfhydryl reagents. Inhibitory pattern of *p*-hydroxymercuribenzoate and phenylglyoxal revealed accessible sulfhydryl and arginyl residue(s) as functional group(s) in the earlier part of electron transport chain as possibly the binding site of NADPH or FAD.

Introduction

The assimilatory nitrate reductase (NADPH : nitrate oxidoreductase, EC 1.6.6.3) from *Neurospora crassa* is responsible for catalyzing the first step in

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the reduction of nitrate to ammonia needed for the ultimate biosynthesis of amino acids and other nitrogen-containing cell constituents. The enzyme was characterized to be a nitrate-inducible, sulfhydryl-containing FAD protein [1]. Molybdenum was shown to be a functional metal constituent [2—4]. Further characterizations of the enzyme [5—7] established: (a) a *b*-type cytochrome (designated cytochrome b_{557} (*N. crassa*)); (b) a proportional relationship of 4 associated activities, namely NADPH-nitrate reductase activity, reduced methyl viologen-nitrate and $FADH_2$-nitrate reductase activities, and NADPH-cytochrome *c* reductase activity; (c) a $s^{0,725}_{20,w}$ value of 7.9 and a molecular weight of 230 000; (d) the selective inhibitory effect on the associated activities by various metal binding agents, which led to the suggestion of the possible involvement of a third metal in addition to molybdenum and heme-iron. An electron transfer scheme was suggested as follows [7]:

$$\text{NADPH} \rightarrow \text{FAD} \xrightarrow{[\text{Metal?}]} \text{cytochrome } b_{557} \rightarrow \text{Mo} \rightarrow NO_3^-$$
$$\downarrow$$
$$\text{cytochrome } c$$

The present paper reports a new preparative procedure to purify *Neurospora* assimilatory nitrate reductase to homogeneity. The determination of subunit composition, amino-terminus, metal content and several other properties of the purified enzyme are included. The possibility of a third metal moiety other than molybdenum and heme-iron was eliminated. A proposed structure for the enzyme and a modified electron transfer pathway is presented. Preliminary accounts of this study have been published [8,9].

Experimental Procedure

Materials

Sources of most substrates, cofactors, and various chemicals have been cited [7]. In addition, the following chemicals were obtained from Sigma: dithiothreitol, imidazole, phenylmethylsulfonylfluoride, streptomycin sulfate, Tris, cacodylic acid, 2(*N*-morpholino)ethane sulfonic acid, *N*-ethylmaleimide, 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide · HCl, thioglycollic acid, Coomassie Brilliant Blue R, phosphorylase *a*. Ampholines were purchased from LKB Instruments; phenylglyoxal from Aldrich; sodium dodecyl sulfate from Matheson, Coleman and Bell; and toluene-3, 4-dithiol, haemin, and all polyacrylamide gel agents from Eastman; trypsin (TRTPCK) and chymotrypsin (CDI) from Worthington. Immunoglobulin was a gift from Dr. John Cebra. Superbrite glass beads (size 0.2 mm) were obtained from Potters Industries (Carlstadt, N.J.).

Culture methods

N. crassa (wild-type STA4) was maintained and grown as reported in earlier studies [7]. The harvested mycelia were stored in small balls at −20°C for as long as 3 months without loss of nitrate reductase activity.

Assay procedures

Protein. Protein was measured by a modification of the method of Lowry et

al. [7,10], using bovine serum albumin as standard. Absorbance at 280 nm was used to monitor column chromatography eluent.

Nitrite. The nitrite resulting from the enzymatic reduction of nitrate in 0.5 ml assay mixture was determined colorimetrically as described by Garrett and Nason [7].

Enzyme assays. NADPH-cytochrome *c* reductase, reduced methyl viologen-nitrate reductase, $FADH_2$-nitrate reductase and NADPH-nitrate reductase activities were assayed at room temperature according to the method of Garrett and Nason [6,7] with slight modification. Bovine serum albumin (200 μg) was included in each assay after the enzyme purification step employing DEAE-Sephadex column chromatography.

Metal determinations

All containers used in metal determination were washed with 20% nitric acid (v/v) and well rinsed with distilled and de-ionized water.

Molybdenum determination. Molybdenum was determined by the method of Cardenas and Mortensen [11] with enzyme samples of about 1 mg.

Heme determination. The heme content of the enzyme was determined by measuring the absolute absorption spectrum of the reduced pyridine hemochromogen derivative of purified NADPH-nitrate reductase, using pyridine hemochromogen derivatives of hemin as standards [12]. Spectra were measured under identical conditions on a Cary 14 spectrophotometer with enzyme samples of 200—400 μg, or hemin of 0.4—1.6 nmol. The mM extinction coefficients: E_{557} = 28.8 mM^{-1} and $E_{557-540}$ = 20.1 mM^{-1}, were used for calculating the heme content in the enzyme.

Non-heme iron. Non-heme iron in the purified samples of NADPH-nitrate reductase containing 100 μg protein per gel was detected by the method described by Brill et al. [13].

Copper and zinc. Copper in 500-μg samples of the purified nitrate reductase was determined by the method of Smith et al. [14] using neocuproine. Zinc was measured by means of microwave excitation emission spectroscopy [15,16] on 5-μl aliquots at a concentration of 1 mg/ml.

Electrophoresis

Analytical polyacrylamide gel electrophoresis. 3 different buffer systems and 3 different gel concentrations were utilized to verify the purity of the native enzymes. The discontinuous system described by William and Reisfield (Tris · HCl/Tris-diethylbarbiturate, pH 8.0) [17] was used without a stacking gel. 2 multiphasic zone systems used were taken from the computer-generated gel electrophoretic systems compiled by Jovin et al. [18], namely No. 1750, a 2(*N*-morpholino)ethanesulfonic acid/cacodylic acid system (pH 6.5) and No. 1995, a 2(*N*-morpholino)ethanesulfonic acid/acetic acid system (pH 7.5). Electrophoresis was performed at 4°C, at 100 V for 20 min and then increased to 200 V. Reduced methyl viologen-nitrate reductase activity was monitored on the gel after electrophoresis by a modification of the method described by Hacklesby and Hageman [19]. The gels were incubated in 6 ml 0.1 M phosphate (pH 7.3)/2 ml 0.01 M methyl viologen/0.4 ml 0.8% sodium dithionite for 4 min and then transferred into 0.1 M $NaNO_3$. Clear band(s) corresponding

to the positions of the enzyme(s) were marked and the gels stained for protein using Coomassie Brilliant Blue R following the method of Weber and Osborn [20].

SDS and 8 M urea polyacrylamide gel electrophoresis. The technique of Fairbank et al. [21] was used. Protein alkylation with *N*-ethylmaleimide was performed following the method of Waxdal et al. [22]. Alkylated protein was treated in the same manner as non-alkylated samples except dithiothreitol was omitted. Bromophenol Blue at 0.005% was added as tracking dye in all samples. Samples containing 10—25 μg of protein were applied to each gel. The proteins were stained as described for the analytical gel.

N-terminal determination

The N-terminal amino acid was analyzed by the procedure of Gary [23]. Performic acid-oxidized nitrate reductase (250 μg) was used for dansylation and hydrolysis. Identification of dansyl amino acid was carried out by thin layer chromatography on polyamide sheets with 3 solvent systems according to the methods of Woods and Wang [24] and Crawshaw et al. [25].

Peptide mapping

The two subunits of *Neurospora* nitrate reductase were sliced separately from preparative SDS gels and eluted electrophoretically from the gel after brief staining. The isolated subunits were verified for contamination by SDS electrophoresis with 7.5% gel. The individual subunits were digested by trypsin or chymotrypsin in the presence of 0.5% SDS, then analyzed by SDS electrophoresis on 15% slab gel. All procedures of Cleveland et al. [26] were followed, with the modifications that during digestion, the protein concentration was 0.2—0.5 mg/ml and the ratio of protease and substrate was approx. 1—5 or —10 (w/w).

Results

Enzyme purification

Unless otherwise indicated, all purification steps were carried out at 0—4°C in the following buffers which always contained 0.5 mM EDTA 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride (to minimize proteolysis): buffer A, 0.02 M phosphate (pH 7.3); buffer B, 0.02 M imidazole-HCl (pH 7.3); buffer C, 0.01 M phosphate (pH 7.0); buffer D, 0.1 M phosphate/0.2 M KBr (pH 7.0); and buffer E, 0.05 M sodium phosphate (pH 6.9). Storage of the enzyme at all purification steps was at —10°C in the indicated buffer which also contained 30% glycerol (v/v), and 0.1 mM FAD.

Extraction of the enzyme: Frozen *N. crassa* (STA 4) mycelia of 600 g were homogenized in 1.5 l buffer A and 600 ml of acid-washed glass beads in a Waring blender for a total of 7.5 min in 1.5-min intervals. The homogenate was filtered through a Miracloth (Chicopee Mills, Midtown, N.J.) and the residue was further rinsed with 300 ml of buffer A to yield a crude extract.

Streptomycin sulfate precipitation: The extract was adjusted to pH 5.9 with 0.5 M acetate buffer (pH 3.9) and freshly prepared 10% aqueous streptomycin sulfate was then added to a final concentration of 2.5—3.0 mg antibiotic/ml.

The resulting mixture was centrifuged at 13 000 × *g* for 25 min and the supernatant solution designated as Streptomycin supernatant and contained 95% of the enzyme activity.

$(NH_4)_2SO_4$ precipitation: Solid $(NH_4)_2SO_4$ at 320 g/l was added to the Streptomycin supernatant. The precipitate containing the enzyme was collected by centrifugation at 13 000 × *g* for 25 min, then dissolved in buffer A to about 1/50 of the original volume.

DEAE-Sephadex column fraction: DEAE-Sephadex A-50 (5.0 × 35 cm) was equilibrated in buffer B/0.22 M KCl. Residual $(NH_4)_2SO_4$ in the sample was removed either by elution through a Sephadex G-50 column (5.0 × 70 cm) or by dialysis against buffer B. Solid KCl was added to the resulting sample to bring the conductivity to that of buffer B/0.22 M KCl, and the preparation was then applied to the DEAE-Sephadex A-50 column. The column was washed with 1.5 bed volume of buffer B/0.22 M KCl resulting in the removal of about 80% of the protein with about 5% loss of enzyme activity, and then eluted with buffer B/0.3 M KCl. Active eluates were pooled, and concentrated by $(NH_4)_2SO_4$ precipitation (320 gm/l). The precipitate was recovered by centrifugation at 20 000 × *g* for 20 min and dissolved in buffer A.

Bio-Gel A-1.5 m gel filtration fractionation. This preparation was applied to a Bio-Gel A-1.5 m column (3.7 × 110 cm) equilibrated in buffer A, and the same buffer used for elution. The peak eluates were combined, precipitated by $(NH_4)_2SO_4$ (320 g/l), centrifuged at 20 000 × *g* for 20 min and the precipitate redissolved in minimum amount of buffer A. The resulting solution was then dialyzed against 3 changes of 100 vols. of buffer A over 2 h.

Isoelectric focusing. A LKB-8100-2 isoelectric focusing column was used with a sucrose density gradient of 2—48% (w/v) and 2% ampholines (pH 4—6). Electrofocusing was conducted for 66 h at 2.7—1.0 W. The active fractions were pooled, precipitated by $(NH_4)_2SO_4$ (320 g/l) and centrifuged at 20 000 × *g* for 20 min. The precipitate was redissolved in minimum volume of buffer C. A Sephadex G-25 column (1.7 × 3.4 cm) equilibrated in buffer C was used to remove $(NH_4)_2SO_4$ and ampholine residues. Dissociation of FAD from the enzyme was usually enhanced by $(NH_4)_2SO_4$ precipitation. At this stage, the enzyme which was stored without adding FAD was estimated to be about 90—95% deflavinized based on its activity without added FAD.

FAD affinity chromatography. The modified procedure of Nishikawa and Bailon [27] was used for the preparation of aminohexanoic-Sepharose. The resulting aminohexanoic-Sepharose was stored at 0—4°C in the presence of 0.5 mM azide. The amount of 6-aminohexanoic acid coupled to the Sepharose beads was monitored by a modification of the method of Naoi and Lee [28] after alkaline hydrolysis. Beads showing less than 6 μmol of aminohexanoic acid per ml gel were not used for FAD coupling. FAD coupling to aminohexanoic-Sepharose was performed as described by Kazarinoff et al. [29].

The resulting material from isoelectrifocusing was applied to the FAD-Sepharose column (1.7 × 13 cm) previously equilibrated in buffer C. Elution was stopped twice for 30 min periods to allow full absorption of the enzymes. The column was washed with at least 2 bed volumes of buffer C followed by 2 bed volumes of buffer D. One bed volume of elution buffer, consisting of 0.1 M phosphate/0.5 M KBr 0.5 mM EDTA 0.5 mM dithiothreitol 0.5 mM

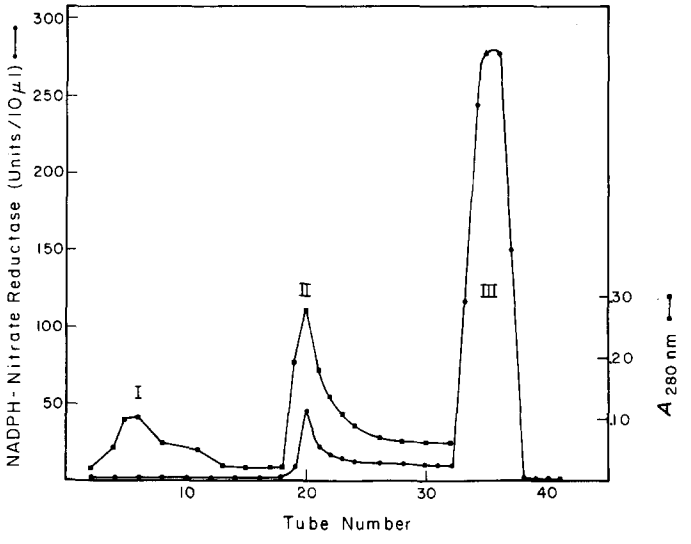


Fig. 1. FAD-Sephadex affinity chromatography of *N. crassa* nitrate reductase. Details are described under Experimental Procedure. (●), NADPH-nitrate reductase activity; (■), absorbance at 280 nm, the fractions containing FAD were not recorded due to interference. I, II, III are protein peaks corresponding to the buffer C and buffer D wash and the final elution.

phenylmethylsulfonylfluoride 1 mM FAD (pH 7.0) was applied. The column flow was stopped, allowed to stand for 8—12 h, and elution resumed with buffer C. Fig. 1 shows a typical elution profile. The pooled active enzyme was concentrated and dialyzed on an Amicon PM-10 Diaflo-membrane under argon against buffer E to give final purified enzyme.

Table I summarizes the pertinent data of the purification procedure. In 5 similar preparations, the resulting purified enzyme had a specific activity ranging from $9 \cdot 10^5$—$1.25 \cdot 10^5$ units per mg of protein, gave a purification of 3000- to 5000-fold and an overall recovery of 25—50%.

Freezing resulted multiple band formation on gels was avoided during the course of purification and storage. Sedimentation coefficient and molecular

TABLE I

SUMMARY OF PURIFICATION OF *N. CRASSA* NADPH-NITRATE REDUCTASE

Fraction	Volume (ml)	Protein		NADPH-nitrate reductase	
		(mg/ml)	Total (mg)	Total activity (unit × 10^{-6})	Specific activity (unit/mg)
1. Crude extract	4775	8.6	40 825	9.91	243
2. Streptomycin sulfate supernatant	4915	3.8	18 825	9.91	527
3. $(NH_4)_2SO_4$ precipitate	102	72.5	7 420	9.57	1 290
4. DEAE-Sephadex A-50	10	75.5	755	7.83	10 365
5. Bio-Gel A-1.5 M	19	20.0	380	5.92	15 584
6. Isoelectric focusing	11.5	3.48	40	3.91	97 825
7. FAD-affinity	3.0	0.80	2.4	3.0	1250 000

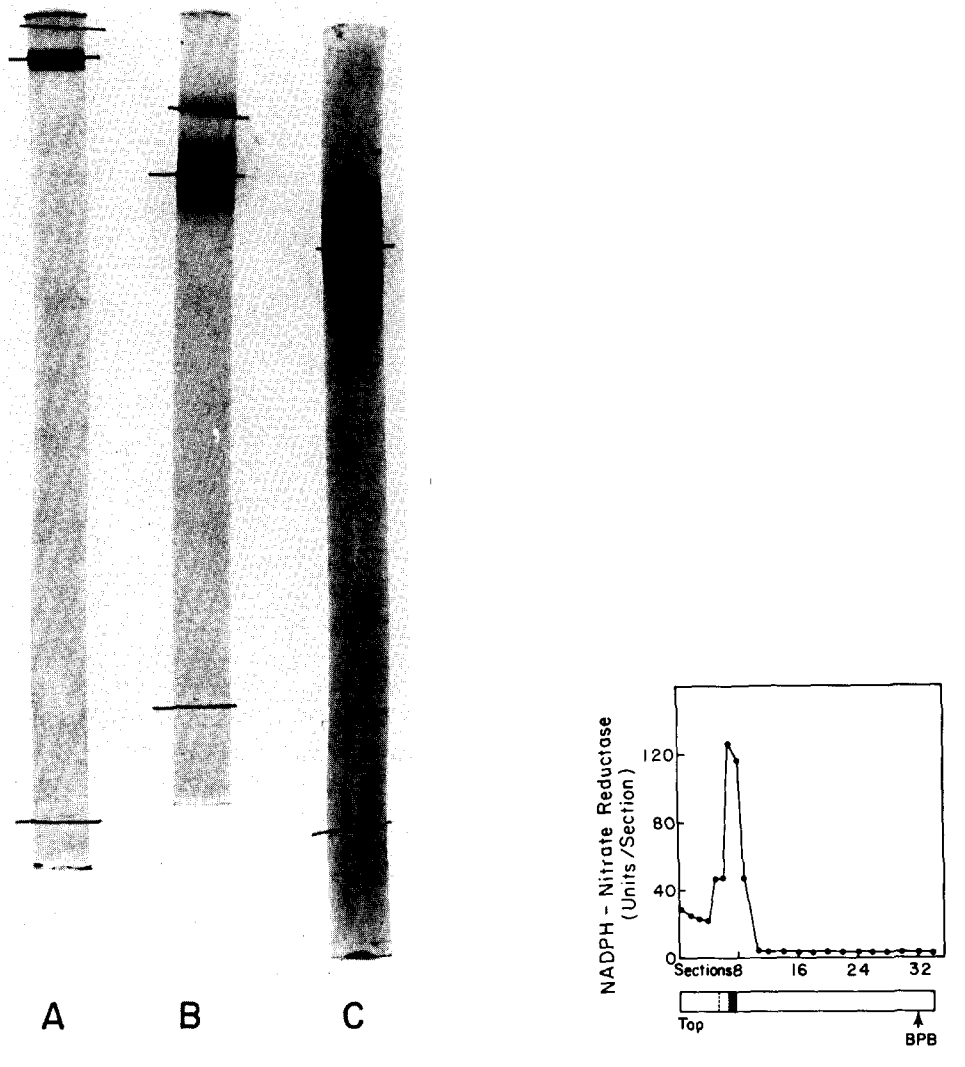


Fig. 2. Polyacrylamide gel electrophoresis of purified NADPH-nitrate reductase (Tris · HCl/diethylbarbiturate system pH 8.0). The gel concentrations were: (A) 7.5, (B) 5.0, and (C) 3.75%. Electrophoresis was carried out as described under Experimental Procedure. A sample of the final purified enzyme at 50 μl (10 to 50 μg, of protein) was used for each gel.

Fig. 3. Distribution of NADPH-nitrate reductase activity after polyacrylamide gel (5%) electrophoresis (Tris · HCl/diethylbarbiturate system pH 8.0). Details are described under Results. NADPH-nitrate reductase activity (●).

weight of the purified enzyme were checked by sucrose density gradient centrifugation and Sephadex G-200 gel filtration and were similar to those reported by Garrett and Nason [7].

Purity of the enzyme

Homogeneity of the purified native enzyme was monitored by several polyacrylamide gel electrophoresis systems. A representative set of the gels of the

Fig. 4. Polyacrylamide gel electrophoresis of SDS and urea treated *N. crassa* nitrate reductase. 7.5% acrylamide was used for all the systems. (A) 1% SDS gel with 1% SDS treated enzyme; (B) 1% SDS/8 M urea gel with 1% SDS/6 M urea-treated enzyme; (C) 1% SDS gel with 1% SDS treated *N*-ethylmaleimide alkylated enzyme. A sample of 10—25 μg of protein was used for each gel.

Tris · HCl/Tris-barbiturate system is shown in Fig. 2. A single intensely stained protein band was obtained at 3 gel concentrations (3.75, 5.0 and 7.5%) after staining with Coomassie Blue. A very faint band was located above the major band in the 5% and 7.5% gels. Both the intense and faint bands showed reduced methyl viologen nitrate-reductase activity. Identical gels (5% concentrations) were sliced into approx. 2.2-mm slices and each slice individually assayed for NADPH-nitrate reductase activity. The distribution of the peak NADPH-nitrate reductase activity among the gel slices coincided exactly with the dark stained protein band the tailing activity corresponded with the faint band (Fig. 3). 2 other electrophoresis sytems gave essentially the same results.

Subunits of the enzyme

Both *N*-ethylmaleimide-alkylated and non-alkylated purified *Neurospora*

nitrate reductase migrated as 2 protein bands on polyacrylamide gel, containing 0.1—2.0% SDS or 8 M urea/1% SDS (Fig. 4). The proportion of the 2 bands (as indicated by the intensity of protein stain) varied from one preparation to another. Each preparation, however, showed consistently the same pattern after several electrophoresis determinations suggesting that the difference among preparations was not introduced by individual electrophoresis. The molecular weights of the two subunits were calculated as 130 000 and 115 000 from 5 determinations using β-galactosidase, phosphorylase *a*, bovine serum albumin and immunoglobulin heavy chain as standards. Prolonged storage of the enzyme (1 year or more) resulted in preparations with decreased intensity of the upper band. Small peptides from possible proteolytic activities were not detected on 15—20% SDS gel.

Metal determination and spectrum

Table II represents a summary of the determinations of molybdenum, heme, and non-heme iron content of the enzyme from 4 different individual preparations. Each value is expressed in mol/mol enzyme, assuming the average molecular weight of nitrate reductase to be 230 000 [7]. The average molybdenum and heme-iron levels were 0.89 mol and 1.54 mol/mol protein, respectively, giving an approximate ratio of 1 : 2. Due to the limited amount of enzyme isolated, we measured molybdenum and heme-iron on a relatively small scale and duplicates were not performed. Non-heme iron was not detected by the α,α-dipyridyl method [13]. Copper and zinc were not found by the methods used.

The spectrum of reduced pyridine hemochromogen derived from purified nitrate reductase was confirmed to be a pyridine derivative of protoporphyrin IX type, with α,β and Soret peaks at approx. 557, 523 and 417 nm in the reduced state. The spectrum of the native enzyme was also confirmed as a *b*-type cytochrome, as reported by Garrett and Nason [6].

N-terminal amino acid

A single dansyl substituted amino acid, that of glutamic acid, was found after chromatography on polyamide sheets.

TABLE II

METAL CONTENT OF HOMOGENEOUS *N. CRASSA* NADPH-NITRATE REDUCTASE

Details of all the analytical methods are given under Experimental Procedures. I, II, III and IV designate 4 different preparations of purified nitrate reductase.

Determination	Experiment				
	I	II	III	IV	Average
	unit/mg protein $\times 10^{-5}$				
Specific activity	12.50	9.60	11.50	9.74	
	mole/mole of protein				
Molybdenum	0.79	0.77	0.89	1.10	0.89
Heme	1.50	1.33	1.69	1.63	1.54
Nonheme iron	n.d. *	n.d.	n.d.	n.d.	None

* Not detectable.

Proteolytic mapping

The 115 000- and 130 000-dalton subunits isolated from pure *Neurospora* nitrate reductase were free from contamination as indicated by SDS-gel electrophoresis. Trypsin and chymotrypsin digestions were carried out at 37°C for 40 min. The resulting peptide patterns from the trypsin digestion shown in Fig. 5 appeared to be similar. No apparent difference was shown with the chymotryptic digestion.

Effect of inhibitors on NADPH-nitrate reductase and associated activities

Inhibition by metal binding agents. An earlier report [7] indicated the possible existence of a third metal component in the enzyme in addition to molybdenum and heme-iron by the study of metal inhibitors on the partially purified enzyme. The effect of metal inhibitors were examined again with the purified enzyme as shown in Table III. Inhibition by cyanide and azide virtually showed the same results. Contrary to their report is about the metal binding agents *o*-phenanthroline and 8-hydroxylquinoline. They found that

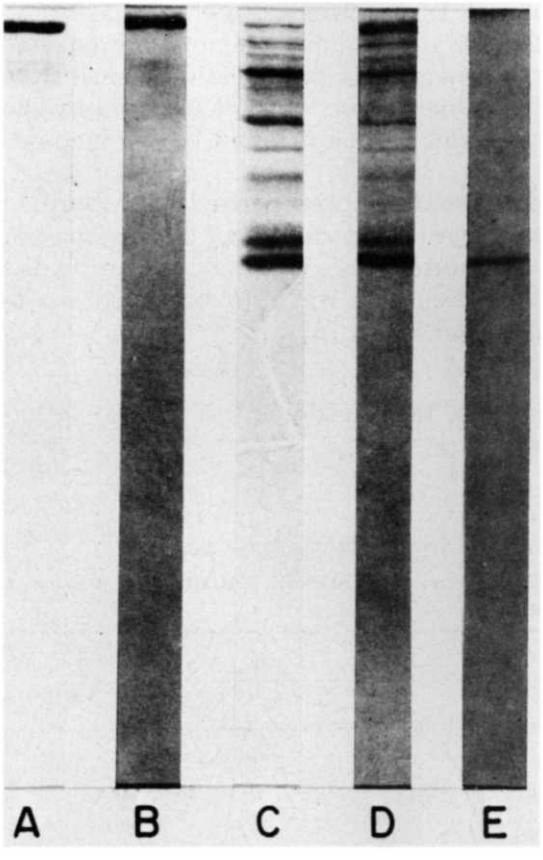


Fig. 5. Tryptic digestion patterns of *Neurospora* nitrate reductase subunits: Details are described under Experimental Procedures. 30 μl of each resulted sample was used for SDS electrophoresis on 15% slab gel. A and B respectively, 115 000- and 130 000-dalton subunits before digestion; C and D, after digestion; E, trypsin.

TABLE III

EFFECT OF METAL BINDING AGENTS ON *N. CRASSA* NITRATE REDUCTASE AND ASSOCIATED ACTIVITIES

The enzymatic activities were measured by the standard procedure, except for the inclusion of the indicated inhibitors. The data are expressed as percentage of the uninhibited rate. Purified enzymes were diluted in 0.1 M phosphate (pH 7.3) at: (a) 1 μg/ml for NADPH-, and reduced methyl viologen-nitrate reductase activities; (b) 3 μg/ml for $FADH_2$-nitrate reductase; and (c) 0.25 μg/ml for NADPH-cytocytochrome *c* reductase. Diluted enzymes were incubated with the inhibitors at the concentrations shown at room temperature for 20 min unless otherwise indicated.

Inhibitor and final concentration	(M)	Inhibition (%)			
		NADPH-nitrate reductase	MVH-nitrate reductase	$FADH_2$-nitrate reductase	NADPH-cytochrome *c* reductase
NaN_3	10^{-4}	22	20	5	0
	10^{-3}	82	54	39	0
	$5 \cdot 10^{-3}$	96	84	59	0
KCN	10^{-4}	3	18	10	0
	10^{-3}	61	47	12	0
	10^{-2}	99	90	72	0
EDTA	$5 \cdot 10^{-3}$	0	12	0	0
	10^{-2}	0	27	0	0
o-phenanthroline or	10^{-3}	0	0	0	0
8-hydroxylquinoline	$5 \cdot 10^{-3}$	0	0	0	0
(in 0.1 M phosphate buffer)	10^{-2}	0	0	0	0
o-phenanthroline	$2.5 \cdot 10^{-3}$	+40 *	40	+80	0
(in 0.1 M phosphate buffer)	$5 \cdot 10^{-2}$	18	49	+68	0
plus 25% ethanol)	10^{-3}	17	46	+39	0
8-hydroxyquinoline	10^{-2}	4	15	20	0
(in 0.1 M phosphate buffer)	$5 \cdot 10^{-3}$	37	15	40	0
plus 25% ethanol)	10^{-2}	94	22	70	0
Enzyme in 0.1 M phosphate	(1 min **)	0	+300	+33	0
buffer plus 25% ethanol	(20 min **)	0	+300	0	15
	(60 min **)	90	+300	—	70

* Positive sign indicates percentage of activity increase.

** Incubation period.

these two agents caused some inhibition of reduced methyl viologen-nitrate and $FADH_2$-nitrate reductase activities with no effect on cytochrome *c* reductase activity. In the current study, the effects of these two inhibitors were only observed when the agents were dissolved in the buffer containing 25% ethanol. When *o*-phenanthroline or 8-hydroxylquinoline were dissolved in regular buffer, no effects on any of the 4 activities were detected. Exposure of the enzyme to ethanol alone resulted in an immediate increase of the reduced methyl viologen-nitrate reductase and $FADH_2$-nitrate reductase activity, and the effect on NADPH-cytochrome *c* reductase and NADPH-nitrate reductase activities were detected after exposure to ethanol for 20 min or longer.

Inhibition of sodium p-hydroxymercuribenzoate and restoration by thiol reagents. The effect of *p*-hydroxymercuribenzoate on all four enzymatic activities of NADPH-nitrate reductase (Fig. 6A) confirms the earlier report [7]. Further study illustrates the reversal of the *p*-hydroxymercuribenzoate inhibitory pattern with thiol reagents. Fig. 6B shows that the addition of 0.1 mM dithiothreitol (0.05 mM for the NADPH-cytochrome *c* reductase) to

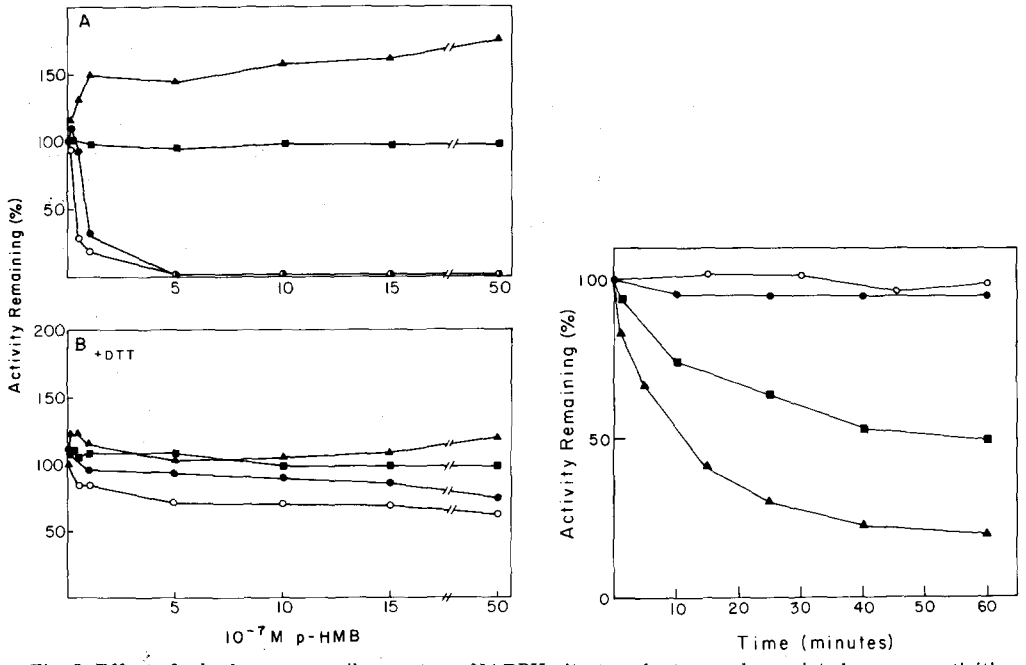


Fig. 6. Effect of *p*-hydroxymercuribenzoate on NADPH-nitrate reductase and associated enzyme activities, and their restoration by dithiothreitol. Purified enzyme (without added FAD) was diluted in 0.1 M phosphate (pH 7.3) same as indicated in Table III. Incubation with *p*-hydroxymercuribenzoate at the indicated concentrations was at 0°C for 10 min. For the restoration, dithiothreitol was added at 0.05 mM for the NADPH-cytochrome *c* reductase, and 0.1 mM for the other three activities, and incubation was continued at 0°C for another 10 min. All activities were assays by the standard procedures. The data are expressed as remaining activity to the untreated protein. NADPH-nitrate reductase activity, (●); reduced methyl viologen-nitrate reductase activity, (▲); $FADH_2$-nitrate reductase activity, (■); NADPH-cytochrome *c* reductase activity (○).

Fig. 7. Effect of phenylglyoxal on NADPH-nitrate reductase activity. Purified enzyme was diluted to 1 μg/ml in 0.1 M phosphate (pH 7.3), incubated at 20°C with (a) 4 mM phenylglyoxal, (▲); (b) 4 mM FAD for 10 min at 0°C then 4 mM phenylglyoxal, (■); (c) no additions, (●); and (d) 4 mM FAD, (○). NADPH-nitrate reductase activity at various time intervals were assayed by standard methods.

the *p*-hydroxymercuribenzoate inhibited enzyme restored the activity of reduced methyl viologen-nitrate reductase to almost 100%, NADPH-nitrate reductase to 75—90%, and NADPH-cytochrome *c* to 60—70%. Similar results were obtained by using 1 mM reduced glutathione.

Inhibition by phenylglyoxal. Arginyl residue(s) involved in the binding sites for anionic nucleotide coenzymes in many enzymes was proposed in recent years [30,31]. Phenylglyoxal was employed for the examination of the possible functional arginyl residues of NADPH-nitrate reductase in the present study. As shown in Fig. 7, phenylglyoxal at 4 mM (or as high as 20 mM) in 0.1 M phosphate (pH 7.3) inactivated the NADPH-nitrate reductase activity to 40% after 15 min incubation, and 20% after 60 min. Partial protection was afforded by preincubating the enzyme with FAD.

Preliminary attempts to distinguish inhibition by *p*-hydroxymercuribenzoate and phenylglyoxal are shown in Table IV. When the enzyme is inhibited 75% by *p*-hydroxymercuribenzoate (line 3), it can be partially protected by

TABLE IV

PROTECTION AND RESTORATION OF THE INHIBITION OF NADPH-NITRATE REDUCTASE BY *p*-HYDROXYMERCURIBENZOATE (*p*-HMB) AND PHENYLGLYOXAL (PG)

Purified enzyme (without added FAD) was diluted in 0.1 M phosphate (pH 7.3) to 1 μg/ml. Inhibitors and reagents at the indicated concentrations were added and incubated with the enzyme in the order listed. All incubations were carried out at 0°C for (a) 10 min for FAD protection or restoration, (b) 10 min for *p*-hydroxymercuribenzoate inhibition, (c) 10 min for dithiothreitol (DTT) restoration, (d) 30 min for phenylglyoxal inhibition. NADPH-nitrate reductase activity was assayed by the standard procedure. The data are expressed as % of remaining activity of the non-inhibited rate.

Enzyme treatment	NADPH-nitrate reductase activity remaining (%)
1. Native enzyme	100
2. + 1 mM FAD	105
3. + 0.6 μM *p*-HMB	26
4. + 1 mM FAD + 0.6 μM *p*-HMB	61
5. + 0.6 μM *p*-HMB + 0.1 mM DTT	95
6. + 0.6 μM *p*-HMB + 4 mM PG	5
7. + 1 μM *p*-HMB	0
8. + 1 μM FAD + 1 μM *p*-HMB	0
9. + 1 μM *p*-HMB + 1 mM FAD	0
10. + 1 μM *p*-HMB + 0.1 mM DTT	74
11. + 4 mM PG (or 20 mM)	24
12. + 1 mM FAD + 4 mM PG	50
13. + 4 mM PG + 1 mM FAD	58
14. + 4 mM PG + 0.6 μM *p*-HMB	0

preincubation with FAD (line 4) and restored by the addition of dithiothreitol (line 5). If total inhibition by *p*-hydroxymercuribenzoate was reached, FAD did not protect or restore any of the activity (lines 7, 8 and 9) while the addition of dithiothreitol restored most of the activity (line 10). As for phenylglyoxal, total inhibition was not obtained by increasing the concentrate of this inhibitor to as high as 20 mM (line 11). FAD (1 mM or 4 mM) offered partial protection and restoration for the inactivation (lines 12 and 13). More important, partially inactivated enzyme by *p*-hydroxymercuribenzoate did not prevent it from further inactivation by phenylglyoxal or vice versa (lines 6, 14).

Discussion

The *N. crassa* assimilatory NADPH-nitrate reductase, now purified 5000-fold, behaves as a homogeneous protein as shown by the single protein band in polyacrylamide gel electrohoresis at pH 6.5, 7.5 and 8.0 (Fig. 2). The purified enzyme remains stable for at least 6 months at −10°C in 0.05 M sodium phosphate (pH 6.9)/30% glycerol 0.5 mM EDTA 0.5 mM dithiothreitol 0.5 mM phenylmethylsulfonylfluoride 0.1 mM FAD.

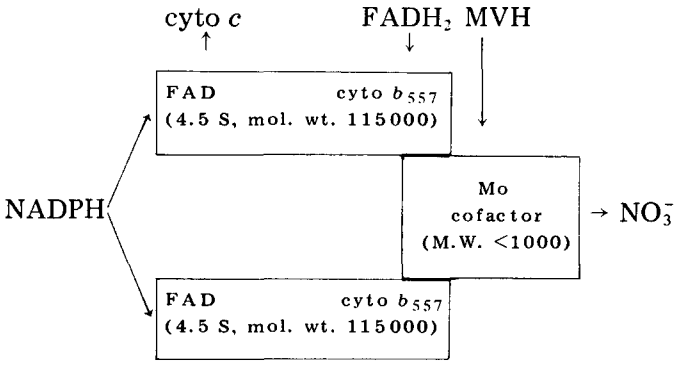
The appearance of two protein bands of molecular weights 115 000 and 130 000 (Fig. 4) upon SDS and urea gel electrophoresis of the purified enzyme (230 000 daltons) seems to suggest two kinds of subunits. However, different preparations of purified enzyme gave variable ratios of the two bands on SDS gels favors the possibility that the two subunits are either derived from a single subunit or interconverted from one to the other. The possibility of artifacts

introduced by purification or uneven SDS binding are not totally ruled out. The single subunit hypothesis is supported by (a) finding one N-terminal amino acid (glutamic acid) for the two subunits; although, the two different subunits may contain the same N-terminus or one of the N-terminus is blocked; (b) proteolytic mapping of the two separated subunits revealed no apparent differences in their digestion pattern. Thus, we tend to believe that the 130 000-dalton subunit is a modified form of the 115 000-dalton subunit, and *Neurospora* nitrate reductase consists of two similar (if not identical) subunits with molecular weight of 115 000. This hypothesis is compatible with our earlier observations [4,32—35] that in vitro assembly of a 7.9-S *Neurospora* nitrate reductase can be obtained from a 4.5-S subunit of the mutant *nit-1* extract and molybdenum-cofactor isolated from a wide range of sources. The *nit-1* protein responsible for the assembly activity and the NADPH-cytochrome *c* reductase activity has been purified to near homogeneity, and shown to be a subunit of approx. 115 000 daltons (Horner, unpublished results). This datum is also supported by the results of Ketchum [37]. We have also isolated the molybdenum cofactor as a highly labile molecule with a molecular weight of less than 1000 (refs. 4 and 35 and unpublished results). The phenomenum of the in vitro assembly of wild-type *Neurospora* nitrate reductase by the incubation of induced *nit-1* extract possessing only the NADPH-cytochrome *c* reductase activity with molybdenum cofactor from extracts of other non-allelic mutants or other sources can be ascribed to the combination of 2 protein subunits from induced *nit-1* by a linkage of the molybdenum cofactor furnished by acid-treated molybdenum-enzymes [35]. This binding produces a second active site around molybdenum, responsible for the other three associated activities using nitrate as the terminal electron acceptor. By analogy, in vivo assembly of the enzyme in the wild type *Neurospora* would occur by a similar process except the cells would provide all the components. Antoine [36] reported a molecular weight of 160 000 for the aberrant nitrate reductase produced by the mutant *nit-3*. Without analysis of the subunit structure, he interpreted that the aberrance of the *nit-3* enzyme is due to the absence of a "controlling protein subunit (the *nit-1* locus)". His assumption does not fit into our data. We feel that an aberration or lesion of part of the protein subunit instead of the loss of an entire subunit is an alternative explanation.

No preferential inhibition of NADPH-cytochrome *c* reductase or NADPH-nitrate reductase activity by *o*-phenanthroline or 8-hydroxylquinoline was observed (Table III), which rules out the suggestion of a third metal component made by previous workers [7]. Their observation apparently was attributed to the mild denaturation of the enzyme from the exposure to ethanol. Analysis of the metal content of the purified enzyme thus far shows only the presence of 0.89 mol molybdenum and 1.54 mol heme-iron per mol protein, which best fits 1 mol of molybdenum and 2 mol of cytochrome b_{557} per mol of enzyme. These data favor a model for a molecule of 2 subunits sharing one molybdenum cofactor, but each subunit containing one cytochrome b_{557}. Ketchum and Downey [37] have claimed that their partially purified *Neurospora* nitrate reductase contained 1.59—1.74 mol molybdenum per mol of enzyme. We feel that their substantially higher results could be attributed to the impurity in their preparation. The FAD content can not be

accurately determined due to its easy dissociation from the enzyme during purification. We assume it is 1 FAD per 1 subunit.

Based on all the data, an electron flow scheme of the *Neurospora* assimilatory nitrate reductase is proposed as follows:



The precise site for the electron donors $FADH_2$ and reduced methyl viologen is not clear. Garrett and Nason [7] proposed that $FADH_2$ probably donates its electrons at or before the cytochrome b_{557} site and reduced methyl viologen prior to the molybdenum site, probably after the cytochrome b_{557} site.

The purification and properties of assimilatory nitrate reductase from other sources have been reported. In a partially purified nitrate reductase of *Aspergillus nidulans*, Downey [38] reported no spectral evidence for a cytochrome, whereas MacDonald and Coddington [39] stated that this enzyme (molecular weight of 190 000 and $s_{20,w}$ value of 7.6) does contain a cytochrome as well as molybdenum and FAD. The nitrate reductase from *Chlorella vulgaris* reported [40] has a somewhat different structure (molecular weight 356 000, and sedimentation coefficient 9.76). They proposed that the *Chlorella* nitrate reductase is composed of 3 subunits of 95 000—115 000 daltons with two separate catalytic centers and each center contains 1 FAD, 1 cytochrome b_{557} and 1 molybdenum. Purified nitrate reductase of *Rhodotorula glutinis* [41] is another enzyme containing molybdenum, cytochrome b_{557} and FAD with a sedimentation coefficient of 7.9 S and molecular weight of 230 000. SDS gel electrophoresis gave also two subunit bands for the enzyme and a molecule of two subunits of the same size with only one heme was proposed.

That the inhibitory pattern imposed by *p*-hydroxymercuribenzoate on the four associated activities of nitrate reductase is reversed by the subsequent addition of thiol reagents (Fig. 5) further supports the suggestion of involving sulfhydryl group(s) in the initial portion of the electron transfer sequence [7]. Modification of arginyl residue(s) of NADPH-nitrate reductase with phenylglyoxal (Fig. 6 and Table IV) revealed that arginyl residue(s) may be also involved at either the NADPH- or FAD-binding site. It seems evident that the inactivation of this enzyme by phenylglyoxal is not a result of modification of sulfhydryl group(s) as in the case of *p*-hydroxymercuribenzoate, because partially inactivated nitrate reductase by one reagent is further inactivated by

the other (Table IV, line 6, 14). FAD protects NADPH-nitrate reductase from inactivation by both reagents (Table IV, lines 4 and 12), which indicates both the sulfhydryl and arginyl residues may be proximal in the tertiary structure of nitrate reductase serving in some role in the early portion of the electron transport chain.

Acknowledgement

We greatly appreciated the work performed by Dr. B.L. Vallee and his associates at the Department of Biological Chemistry, Harvard Medical School for the analysis of zinc by means of microwave emission spectroscopy. We would like to thank Larry Rozanski, David Piccoli and Robert Lipnick for their superb technical assistance. This investigation was supported in part by Research Grant GM-21131 and PCM71-01463 from the National Institutes of Health, United States Public Health Service, and the National Science Foundation, respectively. Contribution No. 940 of the McCollum-Pratt Institute.

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