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PREPARATION AND SOME PROPERTIES OF HOMOGENEOUS *NEUROSPORA CRASSA* ASSIMILATORY NADPH-NITRITE REDUCTASE

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Summary

The *Neurospora crassa* assimilatory NADPH-nitrite reductase (NAD(P)H: nitrite oxidoreductase, EC 1.6.6.4), which catalyzes the NADPH-dependent formation of ammonia from nitrite, has been purified to homogeneity as judged by polyacrylamide gel electrophoresis. The specific activity of the purified enzyme is 26.9 μmol nitrite reduced/min per mg protein, which corresponds to a turnover number of 7800 min^{-1}. The enzyme also has associated NADH-nitrite reductase, NADPH-hydroxylamine reductase and NADH-hydroxylamine reductase activities. The stoichiometry of 3 mol NADPH oxidized per mol nitrite reduced and ammonia formed has been confirmed.

The visible absorption spectrum of the nitrite reductase reveals maxima at 280, 390 (Soret) and 580 (α) nm. The latter bands are indicative of the occurrence of siroheme as a prosthetic group. The A_{280nm}/A_{390nm} ratio of 7.0 and the Soret/α ratio of 3.8 are compatible with values reported for other purified siroheme-containing enzymes.

These results are discussed in terms of the comparative biochemistry of various enzymes involved in nitrite, hydroxylamine and sulfite metabolism in *Neurospora crassa* and other organisms.

Introduction

The assimilatory nitrite reductase (NAD(P)H:nitrite oxidoreductase, EC 1.6.6.4) from *Neurospora crassa* catalyzes the NADPH-dependent reduction of nitrite to ammonia, a 6-electron transfer reaction [1,2]. Previous studies from this laboratory [3,4] have demonstrated that this enzyme is an FAD-dependent, siroheme-containing protein of M_r = 290 000. Chemical, spectral and kinetic studies indicate that the siroheme moiety is intimately involved in the catalytic function and may provide the nitrite-binding site within this enzyme [4]. The FAD component precedes siroheme in the electron transfer sequence

as judged by the FAD-dependency for reduction of the siroheme by NADPH and the fact that the siroheme-independent NADPH-diaphorase activity found with this nitrite reductase requires FAD [1,4]. These and other considerations suggest the reaction series: NADPH → FAD → siroheme → NO_2^-. In addition to nitrite, this enzyme is also capable of reducing hydroxylamine. Further, NADH is an effective electron donor in place of NADPH in either a diaphorase assay or in nitrite or hydroxylamine reduction [1,3]. Because of the functional diversity implicit in this multiplicity of electron transfer reactions, especially the overall 6-electron reduction of nitrite to ammonia, a precise description of the physical, chemical and structural properties of this nitrite reductase and the relationships of these properties to function would be most interesting. Until recently, such considerations have been hampered by the combined instability and impurity of available *N. crassa* nitrite reductase preparations [3,4].

This paper reports the purification of the *N. crassa* nitrite reductase to homogeneity. In addition, several properties of the nitrite reductase, including its absorption spectra and the reaction stoichiometry, have been studied using pure enzyme.

Methods

Growth of mycelia. N. crassa mutant *nit*-1 (a nitrate reductase-deficient strain) was grown as described previously [4].

Assay procedures. Protein concentrations were determined by a modified Lowry method using crystalline bovine serum albumin as the standard [5]. Initial concentrations of the bovine serum albumin were determined spectrophotometrically at 280 nm (the extinction coefficient of bovine serum albumin was taken as 0.67 ml · mg^{-1} · cm^{-1}) [6]. In the purified samples (Blue Dextran-Sepharose 4B eluates), it was first necessary to precipitate the protein by addition of 0.025 ml 2% sodium deoxycholate/ml cold 24% trichloroacetic acid [7]. This procedure was necessary to eliminate interferences caused by the nucleotides and other substances routinely added to the buffers. After precipitation, the modified Lowry procedure was followed [5].

A modified version of the method of Nicholas and Nason [8] was used to determine nitrite concentrations. To a 1.5-ml sample containing 10—50 nmol nitrite, 0.5 ml 1% (w/v) sulfanilic acid in 20% (w/v) HCl was added and mixed. 0.5 ml 0.12% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride was added and the solutions mixed. After 10 min, the absorbance was determined on a Klett-Summerson colorimeter equipped with a No. 54 filter.

Ammonia determinations were made following diffusion of NH_3 from alkaline solution and collection on acid-coated glass rods using a modified version of the phenol-hypochlorite method [9]. Samples containing 0—60 nmol ammonia in 1 ml were placed in 3 × 7 cm vials and 1.5 ml saturated Na_2CO_3 were added to each. Rubber stoppers holding ground glass rods dipped in 0.25 M H_2SO_4 were quickly inserted to seal each vial. The stoppered vials were placed horizontally into a vertical pin wheel which was rotated for 30 min. The rods were then removed and dipped into 1 ml deionized water in shell vials. Then 0.5 ml each color reagent was added in the following order: 2.5% (w/v) phenol in 1.5% (w/v) NaOH; 0.0005% (w/v) sodium nitroprusside; 50 mM

Na_2CO_3; and a 1 : 20 dilution of Clorox (sodium hypochlorite). Color was allowed to develop for 30 min in the dark and the absorbance was read at 625 nm on a Gilford 2400 spectrophotometer.

Enzyme assays. NAD(P)H-nitrite reductase: Pyridine nucleotide-dependent nitrite reductase was determined by three methods: (1) Nitrite-dependent pyridine nucleotide oxidation was followed at 340 nm, as previously described [3]. The number of nmol NAD(P)H oxidized/min divided by three (it will be shown in Results that 3 mol NAD(P)H are oxidized per mol nitrite reduced) gave nmol nitrite reduced/min. Specific activity is defined as μmol nitrite reduced/min per mg protein. (2) Pyridine nucleotide-dependent nitrite disappearance was measured colorimetrically by a slight modification of previous procedures [3]. The assays were run in test tubes at room temperature using 1 ml reaction mixture which contained 0.2 ml 0.5 mM $NaNO_2$, 0.1 ml 0.1 mM FAD, 0.1 ml 1 M potassium phosphate buffer (pH 7.0), deionized water and enzyme sufficient to catalyze the disappearance of 10—100 nmol nitrite in 5—10 min. Addition of 0.1 ml 2 mM NAD(P)H initiated the reaction and at the desired time, addition of 0.02 ml 0.1 M KCN was used to stop the reaction. Because high concentrations of pyridine nucleotides interfere with the color development, they were precipitated by addition of 0.2 ml 25% (w/v) barium acetate and 1.5 ml 95% ethanol. After mixing, the samples were placed in ice for 5—10 min and then centrifuged for 20 min. A 1.5-ml aliquot of the supernatant was used for the nitrite determination, as described above. One activity unit is defined as the disappearance of 1 μmol nitrite/min. (3) Ammonia formation from nitrite was measured in 1 ml reaction mixture identical to that used in measuring nitrite disappearance. Sufficient enzyme to catalyze the formation of 10—100 nmol ammonia in 5—10 min was included. As in the nitrite disappearance assay, addition of 0.1 ml 2 mM NAD(P)H initiated the reaction and addition of 0.02 ml 0.1 M KCN stopped the reaction. Ammonia concentration was immediately determined using the diffusion technique described earlier. One unit of activity is defined as the formation of 1 μmol ammonia/min.

NAD(P)H-hydroxylamine reductase: Pyridine nucleotide-dependent hydroxylamine reductase activity was measured by two different procedures: (1) The assay procedure for hydroxylamine-dependent pyridine nucleotide oxidation was described previously [3]. 1 activity unit is defined as the oxidation of 1 μmol NAD(P)H/min. (For each mol NAD(P)H oxidized, 1 mol hydroxylamine is reduced). (2) Hydroxylamine-dependent ammonia formation was assayed using the same reaction mixture as the hydroxylamine-dependent pyridine nucleotide oxidation assay. Immediately after terminating the reaction with 0.02 ml 0.1 M KCN, the samples were processed for ammonia determination according to the phenol-hypochlorite method previously described. One unit of activity is defined as the formation of 1 μmol ammonia/min.

Stoichiometry. The stoichiometry of the NAD(P)H-nitrite reductase reaction was determined in 10-ml reaction mixtures containing 100 μmol potassium phosphate buffer (pH 7.0), 1.0 μmol $NaNO_2$, 100 nmol FAD, deionized water and sufficient enzyme to cause the disappearance of 300—600 nmol nitrite in 9 min. The reaction was initiated by adding 2 μmol NAD(P)H. A 2.5-ml aliquot was immediately added to a test tube containing 0.025 ml 0.1 M KCN to stop the reaction. Using this zero time sample as a reference and an aliquot of the

remaining sample as the experimental, the reaction rate was recorded (measuring the oxidation of NAD(P)H at 340 nm) for 9 min. Also, 2.5-ml aliquots of the reaction mixture were removed every 3 min and placed in test tubes containing 0.025 ml 0.1 M KCN. Determinations of nitrite and ammonia concentrations by the described procedures were performed immediately after the 9-min sample was stopped.

The procedure for the determination of the NAD(P)H-hydroxylamine reductase reaction was identical to the previous procedure except that 1 mmol potassium phosphate buffer (pH 8.0) was used in place of 100 μmol pH 7.0 buffer and 120 μmol hydroxylamine were used instead of nitrite.

Preparation of Blue Dextran-Sepharose 4B. Blue Dextran-Sepharose 4B resin was prepared as described by Ryan and Vestling [10].

Results

Purification of NADPH-nitrite reductase

In a typical preparation, 600 g frozen *nit*-1 *N. crassa* mycelia were homogenized in a 4 l stainless steel Waring blendor, operated at low speed with 2400 ml preparation buffer (0.1 M potassium phosphate buffer (pH 7.5)/5 mM EDTA/5 mM cysteine/1 mM phenylmethylsulfonyl fluoride/10% glycerol/10 μM FAD) and 1200 g (2 g/g) cold acid-washed glass pavement marking beads (Minnesota Mining and Manufacturing Co). Homogenization was stopped when the temperature of the homogenate reached −3°C. All purification steps after homogenization were performed at 0—4°C; all centrifugations were 20 min at 27 000 × *g*. The crude homogenate was centrifuged and the resulting supernatant containing 5.1 mg/ml protein (spec. act. 0.025) was designated the crude extract (Fraction 1).

The crude extract was treated with $(NH_4)_2SO_4$ such that the precipitate forming between 40 and 60% saturated $(NH_4)_2SO_4$ could be collected and resuspended in preparation buffer minus FAD (Fraction 2). This step resulted in a 2.8-fold purification (spec. act. 0.07) and a recovery of 165% of the original activity. The increase in activity may be due to the removal of an inhibitor in the crude extract or to a concentration-dependent change in enzyme activity or stability.

Nucleic acids were precipitated from Fraction 2 by treatment with polyethylene glycol 6000 (30%, w/w), followed by dialysis of the supernatant against 10 mM potassium phosphate (pH 7.0)/5 mM EDTA/5 mM cysteine/0.1 mM phenylmethylsulfonyl fluoride/10 mM $Na_2S_2O_4$ to remove $(NH_4)_2SO_4$ and polyethylene glycol. This dialyzed supernatant (Fraction 3) contained 68% of the original activity.

Fraction 3 was then chromatographed on a DEAE-cellulose column (5 × 15 cm; bed volume 300 ml) equilibrated with the previous buffer (phenylmethylsulfonyl fluoride concentration increased to 1 mM). The column was eluted with a linear phosphate gradient composed of 800 ml each of 10 mM and 400 mM phosphate buffer (pH 7.0) containing 5 mM EDTA/5 mM cysteine/1 mM phenylmethylsulfonyl fluoride/10% glycerol/10 μM FAD. The most active fractions were combined and designated the pooled DEAE eluates (Fraction 4). This fraction contained 50% of the original activity of the crude extract and

TABLE I

SUMMARY OF PURIFICATION ON NADPH-NITRITE REDUCTASE

Fraction	Volume (ml)	Total protein (mg)	Total activity (units) **	Specific activity (units/mg)	Recovery (%)	Purification
1. Crude extract	2200	11 200	280	0.025	100	
2. 40—60% *$(NH_4)_2SO_4$ precipitate	200	6 620	463	0.07	165	2.8
3. Dialyzed polyethylene glycol-treated samples	270	2 700	189	0.07	68	2.8
4. Pooled DEAE eluates	248	496	139	0.28	50	11.2
5. 60% $(NH_4)_2SO_4$ precipitate	8	336	97	0.29	35	11.6
6. 40—45% $(NH_4)_2SO_4$ precipitate *	1.6	235	108	0.46	39	18.4
7. Pooled Sepharose 4B eluates	14.5	39	23.4	0.60	8	24
8. Pooled Blue Dextran-Sepharose 4B eluates	12.5	1.0	16.1	16.1	5.8	644
9. Most active Blue Dextran-Sepharose 4B fraction	2	0.2	5.38	26.9	2	1076

* Only 1/3 of Fraction 6 was used for subsequent steps.

** unit = μmol NO_2^- reduced/min per ml.

represented an 11.2-fold purification (spec. act. 0.28).

The 60% saturated $(NH_4)_2SO_4$ precipitate of Fraction 4 was dissolved in preparation buffer and designated Fraction 5.

The precipitate formed by dialysis of Fraction 5 for 3 h against 40% saturated $(NH_4)_2SO_4$ buffer (preparation buffer plus 10 mM $Na_2S_2O_4$) was discarded and the supernatant was then dialyzed for 4 h against 45% saturated $(NH_4)_2SO_4$ buffer/10 mM $Na_2S_2O_4$. The 40—45% saturated $(NH_4)_2SO_4$ precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.0)/5 mM EDTA/5 mM cysteine/1 mM phenylmethylsulfonyl fluoride and represented 39% of the starting activity and an 18.4-fold purification (Fraction 6, spec. act. 0.46).

At this stage of purification, 1 ml Fraction 6 was dialyzed against the previous buffer for 2 h and stored at −70°C for later use. The remaining 0.5 ml of enzyme (representing 33% of the preparation) was immediately chromatographed on a Sepharose 4B column (0.5 × 50 cm; bed volume 90 ml), equilibrated and eluted with the previous buffer. The most active fractions were combined and designated as the pooled Sepharose 4B eluates (Fraction 7) which represented an overall recovery of 8% (but 24% assuming the whole preparation had been used) and a 24-fold purification (spec. act. 0.60).

Fraction 7 was immediately chromatographed on a 3 ml (1.4 × 1.9 cm) Blue Dextran-Sepharose 4B column equilibrated with 10 mM potassium phosphate buffer (pH 7.0)/5 mM EDTA/5 mM cysteine/1 mM phenylmethylsulfonyl fluoride/2 mM Na_2SO_3/10% glycerol. The column was sequentially washed with 15 ml equilibration buffer containing 1 mM ATP and 1 mM $NADP^+$, 15 ml 30 mM potassium phosphate buffer (pH 7.0) and 15 ml equilibration buffer. The nitrite reductase was then eluted with 15 ml equilibration buffer containing

1 mM NADPH to yield the final fraction, pooled Blue Dextran-Sepharose 4B eluates (Fraction 8). This fraction represented recovery of 5.8% and a 644-fold purification. The most active eluate represented a 1076-fold purification (spec. act. 26.9) and 2% recovery.

The results are from the best experiment to date and are summarized in Table I. However, the results are representative and specific activities of 13.3 or more were commonly obtained. Polyacrylamide gel electrophoresis of Fraction 8 revealed the presence of a single protein band, and this band displayed nitrite reductase activity when assayed in situ by incubating the gel for 5 min in a solution containing 0.5 mM methyl viologen, 5 mM $NaNO_2$, and 0.8% $Na_2S_2O_4$. The gel was counterstained with 2.5% tetrazolium red. The protein and activity bands can be seen in Fig. 1.

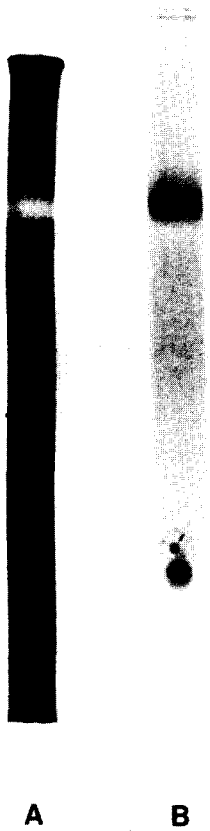

Fig. 1. Polyacrylamide gel electrophoresis of Blue Dextran-Sepharose 4B eluates (Fraction 8) on 5% gels prepared according to Clark [34]. The electrophoresis was performed for 3 h at 4°C and 1.5 mA/tube. Discontinuous Tris/glycine buffer system was used. To 0.20 ml enzyme was added 0.05 ml 1.0% (w/v) bromphenol blue/10% (w/v) sucrose solution. A 0.2 ml (10 μg protein, 0.26 activity unit) aliquot of this mixture was layered onto each gel (15 cm length). Gel A was stained for dithionite-nitrite reductase activity in a reaction mixture containing 0.5 mM methyl viologen, 5 mM NO_2^-, and 0.8% dithionite. The gel was counter-stained with 2.5% tetrazolium red. Gel B was stained for protein with Coomassie Blue R250. The material visible in the lower part of the gels is the Bromophenol Blue dye front.

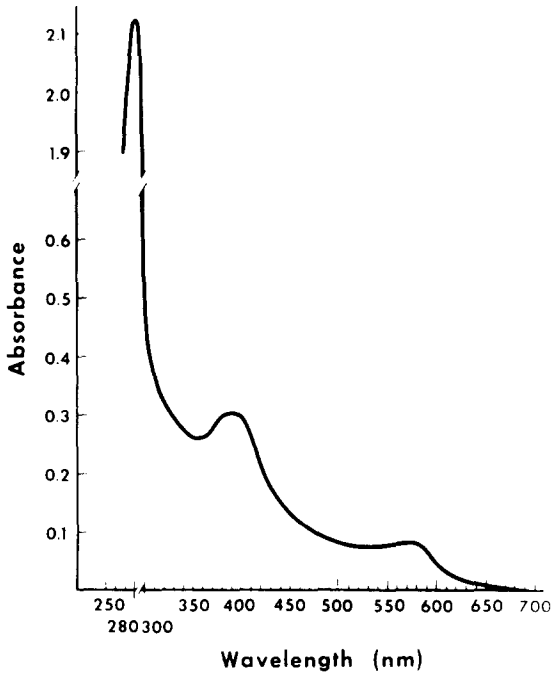

Fig. 2 The absorption spectrum of homogeneous nitrite reductase (Fraction 8, 1.9 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.5) was measured on a Cary 14 spectrophotometer.

Some properties of NADPH-nitrite reductase

Spectrum. The absorption spectrum of homogeneous nitrite reductase was determined on an enzyme sample obtained by pooling eluates from several Blue Dextran-Sepharose 4B columns and dialyzing them for 6 h against 0.1 M potassium phosphate buffer (pH 7.0) saturated with $(NH_4)_2SO_4$. Dialysis was performed in this manner to concentrate the protein and to rid the sample of NADPH which binds to nitrite reductase. The supernatant obtained after centrifugation was discarded and the precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer for 3 h. The spectrum of this sample (protein concentration 1.9 mg/ml) was then recorded, using dialysis buffer as the blank. As shown in Fig. 2, absorption maxima were recorded at 280, 390 (Soret) and 580 (α) nm. The A_{280nm}/A_{390nm} absorbance ratio is 7.0 and the Soret/α ratio is 3.8. The molar extinction coefficient at 390 nm was determined to be $4.65 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

Stoichiometry. A summary of the results of stoichiometry studies is presented in Table II. Blue Dextran-Sepharose 4B eluates (Fraction 8) were always used in these studies. The ratio of NAD(P)H oxidized to nitrite reduced and ammonia formed was 3 : 1 : 1, as demonstrated earlier by Lafferty and Garrett [3] using partially purified enzyme. The ratio of NAD(P)H oxidized to ammonia formed for the hydroxylamine-dependent reaction was 1 : 1. The amount of hydroxylamine reduced was not determined due to the small amounts disappearing. Controls included reaction mixtures minus enzyme, FAD, or nitrite. No NAD(P)H was oxidized, no nitrite was reduced nor was any ammonia formed in any of the controls.

TABLE II

STOICHIOMETRY OF THE NITRITE REDUCTASE ACTIVITIES

The stoichiometry of the nitrite reductase was studied using the assays described in Methods. The Blue Dextran-Sepharose 4B samples (Fraction 8) used in the NADPH-nitrite and hydroxylamine reductase assays contained 0.7 μg protein with a specific activity of 11.3 units/mg. The samples used in the NADH-nitrite and hydroxylamine reductase assays contained 1.4 μg protein with the same specific activity as above. (In the absence of enzyme, nitrite, hydroxylamine or FAD, there was no activity).

Reaction Incubation time (min)	NAD(P)H oxidized (nmol)	NO_2^- reduced (nmol)	NH_3 produced (nmol)	Ratio $\frac{NAD(P)H}{NO2}$	Ratio $\frac{NAD(P)H}{NH_3}$
NADPH-nitrite reductase					
3	59.7	20.0	20.5	3.0	2.9
6	106.5	36.0	36.1	3.0	3.0
9	145.2	51.2	44.1	2.8	3.3
NADH-nitrite reductase					
3	62.6	21.5	24.0	2.9	2.6
6	109.7	32.8	42.0	3.3	2.6
9	146.8	42.6	54.1	3.4	2.7
NADPH-hydroxylamine reductase					
3	80.6		78.2		1.0
5	113.7		104.3		1.1
8	159.7		122.3		1.1
NADH-hydroxylamine reductase					
3	64.5		53.3		1.2
5	95.2		79.5		1.2
8	129.0		105.9		1.2

Molecular activity. The homogeneous nitrite reductase has an activity of 26.9 μmol nitrite reduced/min per mg protein (Table I). Therefore, one molecule of nitrite reductase (M_r = 290 000) [3] can reduce 7800 molecules of nitrite/min under the assay conditions employed. That is, the molecular activity (turnover number) of the *N. crassa* NADPH-nitrite reductase is 7800. In terms of katals, the activity unit recommended by the Enzyme Commission, 1 mol enzyme possesses an activity of 130 katals (mol nitrite reduced/s).

Activities of nitrite reductase. The *N. crassa* nitrite reductase is capable of displaying the following activities in vitro: (1) NADPH-nitrite reductase, (2) NADH-nitrite reductase, (3) NADPH-hydroxylamine reductase, and (4) NADH-hydroxylamine reductase. All of these activities were assayed using pure enzyme (Table III). The preferred electron donor for both the nitrite-depen-

TABLE III

ACTIVITIES AND RATIOS OF ACTIVITIES OF NITRITE REDUCTASE

$\frac{\text{NADPH-nitrite reductase}}{\text{NADH-nitrite reductase}} = 2.3$; $\frac{\text{NADPH-hydroxylamine reductase}}{\text{NADH-hydroxylamine reductase}} = 1.9$.

Activity	nmol pyridine nucleotide oxidized/min/per ml
NADPH-nitrite reductase	7420
NADH-nitrite reductase	3325
NADPH-hydroxylamine reductase	9800
NADH-hydroxylamine reductase	5160

dent and the hydroxylamine-dependent reactions is NADPH. It should be noted that the ratio of the NADPH-dependent activity to the NADH-dependent activity did not change significantly when hydroxylamine was used as the substrate instead of nitrite.

Discussion

The *N. crassa* assimilatory nitrite reductase has been purified to homogeneity as judged by polyacrylamide gel electrophoresis (Fig. 1). The appearance of a single band in gels stained for methyl viologen-nitrite reductase activity which corresponded to the banding pattern in gels stained for protein by Coomassie Blue R-250 warrants this conclusion. Attempts to slice gels and assay the fractions for NADPH-nitrite reductase activity were unsuccessful.

The purified nitrite reductase (Fraction 8) reported in this paper (Table I) has specific activities up to 37 times higher than the partially purified fractions reported by Lafferty and Garrett [3] for their 90-fold purified enzyme. Their purification procedure included ammonium sulfate fractionations, phase separation by polyethylene glycol, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. Until recently, the extreme in vitro lability of the nitrite reductase had limited further attempts at purification and characterization. The increased recovery and high specific activities reported in this paper are, at least in part, a result of including both FAD and dithionite in most buffers. Presumably, dithionite keeps the enzyme in a more stable, reduced state, and in addition, generates sulfite in the buffers which can bind to the active site of the enzyme, thus protecting it from deleterious changes. Sulfite is a competitive inhibitor of the *N. crassa* nitrite reductase with respect to nitrite [11]. While FAD is essential for enzymatic activity, it apparently dissociates easily from the enzyme during the purification procedure. The addition of glycerol to many of the buffers has also helped in stabilizing the enzyme during purification, as well as being essential for maintenance of activity upon storage. Phenylmethylsulfonyl fluoride, a serine protease inhibitor, has reduced the loss of activity during certain early purification steps.

Even with the increased stability acquired by the above-mentioned additions to the buffers, purification of the nitrite reductase to homogeneity depended upon the use of Blue Dextran-Sepharose 4B affinity chromatography. It has been proposed that Blue Dextran complexes with proteins possessing a supersecondary structure called the dinucleotide fold [12] which consists of about 120 amino acids arranged in a β-sheet core of 5 or 6 parallel strands connected by α-helical intrastrand loops located above and below the β-sheet [13,14]. This nucleotide fold is known to form the NAD-binding site in lactate [15], malate [16] and glyceraldehyde phosphate [17] dehydrogenases, as well as the ATP-binding site in phosphoglycerate kinase [13] and adenylate kinase [18]. Apparently, Blue Dextran also has a high affinity for this nucleotide fold and thus, Blue Dextran-Sepharose 4B can serve as an affinity matrix for proteins with this structure. Blue Dextran-Sepharose 4B affinity columns gave up to 50-fold purifications over the previous step (Table I). As discussed in Results, the columns were washed with ATP, $NADP^+$ and phosphate before eluting the nitrite reductase with 1 mM NADPH. Each of the washes eluted some protein.

Due to the variability of these columns, the fractions from each Blue Dextran-Sepharose 4B column were routinely checked for purity by polyacrylamide gel electrophoresis.

Inactivation of enzymatic activity on preincubation with reductants is a property of a number of nitrate assimilatory enzymes, including nitrate reductases from *Chlamydomonas reinhardi* [19], *Chlorella fusca* [20] and spinach [21], and nitrite reductases from *Escherichia coli* [22], *Azotobacter chroococcum* [23] and *N. crassa* [11]. Obviously, elution of the Blue Dextran-Sepharose 4B columns with NADPH exposed the enzyme to such a reductant. Competitive inhibitors of the nitrite reductase protect the enzyme against this inactivation [11]; consequently, 2 mM sulfite was included in elution buffers.

The results of experiments on the stoichiometry of the nitrite reductase reaction essentially confirmed previous observations of Lafferty and Garrett [3]. That is, the ratio of NAD(P)H oxidized to nitrite reduced and ammonia formed was 3 : 1 : 1, as expected for this six-electron transfer reaction. Hydroxylamine can be used in vitro in place of nitrite in a 2-electron transfer reaction also giving ammonia as the product. Lafferty and Garrett [3] found that FAD was necessary for maximal activity; in this study using pure enzyme, no NAD(P)H-nitrite reductase reaction occurred if FAD was absent.

The *N. crassa* nitrite reductase complex has a number of associated activities: NADPH-nitrite reductase activity, NADH-nitrite reductase activity, NADPH-hydroxylamine reductase activity. NADH-hydroxylamine reductase activity and dithionite-nitrite reductase activity [3]. All of the above activities, except the dithionite-nitrite reductase, were assayed using pure enzyme (Table III). The reaction velocities with the two reduced pyridine nucleotides differed, with NADPH giving rates twice as fast as NADH in both the nitrite reductase and hydroxylamine reductase activities, indicating NADPH is the preferred electron donor.

One of the most significant aspects of the *N. crassa* nitrite reductase complex is its ability to catalyze a 6-electron transfer reaction. Only sulfite reductases [24—26] and nitrogenases [27] are known to share this capability, and these proteins are hetero-multimeric proteins. The *N. crassa* nitrite reductase is presumably a dimer of identical subunits [28].

A number of similarities, in addition to catalyzing six-electron reductions, exist between nitrite reductases and sulfite reductases (Table IV). In fact, *E. coli* sulfite reductase was originally classified as a nitrite reductase [29] because of its ability to reduce nitrite and hydroxylamine to ammonia. The sulfite reductases from spinach [25], yeast [26], *E. coli* [30] and *Salmonella typhimurium* [31] can all reduce nitrite and/or hydroxylamine to ammonia. However, the K_m values of the sulfite reductases for nitrite (and hydroxylamine) are too high to permit nitrite to serve as a substrate under physiological conditions [32]. Organisms which can assimilate both sulfate and nitrate thus appear to possess distinct sulfite and nitrite reductases. It is interesting to note that nitrite reductases are not capable of reducing sulfite.

Of interest also is a comparison of the enzymes from photosynthetic and non-photosynthetic organisms. Both the spinach nitrite reductase (M_r = 61 000) and sulfite reductase (M_r = 84 000), as well as the *C. fusca* nitrite reductase (M_r = 63 000), utilize reduced ferredoxin as the physiological electron donor

TABLE IV

COMPARISON OF ASSIMILATORY NITRITE AND SULFITE REDUCTASES

Fd, ferredoxin; MV, methyl viologen; BV, benzyl viologen; PCMB, *p*-chloromercuribenzoate; PHMB, *p*-hydroxymercuribenzoate.

	Nitrite reductase				Sulfite reductase	
	N. crassa [a]	*Chlorella fusca* [b]	Spinach [c]	*Achromobacter Fischeri* [d]	Spinach [e]	*E. coli* [f]
Molecular weight	290 000	63 000	61 000	95 000	84 000	670 000
Electron acceptors	NO_2^-, NH_2OH	NO_2^-, NH_2OH	NO_2^-, NH_2OH	NO_2^-, NH_2OH	SO_3^{2-}, NH_2OH	SO_3^{2-}, NO_2^-, NH_2OH
Electron donors	NADPH, NADH, dithionite	Fd, MV	Fd, MV	NADH, BV, NADPH	Fd, MV	NADPH, MV
Specific activity	26.9 μmol NO_2^-/mg	51.7 units/mg	108 units/mg	1500 units/mg	2.32 μmol MV/mg	2.87 μmol NADPH/mg
Turnover number (min^{-1})	7800	3257	6588	14 250	195	1923
Prosthetic group						
flavin	+	—	—	+	—	+
Non-heme iron	+	?+	+	—	?+	+
Heme	Siroheme	?	Siroheme	Cytochrome *c*	+	Siroheme
Spectral properties						
Absorption maxima	280, 390, 580	278, 384, 573, 630, 692	276, 386, 573 690	278, 409, 525	279, 385, 404, 589 700	278, 386, 587, 714, 455
Absorption ratio 280 nm/390 nm	7.0	3.3	1.8	0.71	—	3.56
Soret/α ratio	3.8	4.1	3.66	7	4.1	4.4
E(Soret) ($M^{-1} \cdot cm^{-1}$)	$4.65 \cdot 10^4$	$2.2 \cdot 10^4$	$3.97 \cdot 10^4$	$1.66 \cdot 10^5$	—	$3.1 \cdot 10^5$
Inhibitors	CO, CN, SO_3^{2-}, arsenite, 8-hydroxyquinoline, PMCB	CO, CN^-, *o*-phenanthroline, 8-hydroxyquinoline, PCMB	CO, CN^-, SO_3^{2-}, PCMB	PHMB, CO, CN^-	CO, PCMB	CN^-, CO arsenite

[a] This paper, refs. 1—4 and 11.
[b] Ref. 35.
[c] Ref. 36.
[d] Ref. 33
[e] Refs. 25 and 37.
[f] Refs. 38 and 39.

and all are of relatively low molecular weight. The enzymes from the non-photosynthetic organisms such as *N. crassa* nitrite reductase (M_r = 290 000) and *E. coli* sulfite reductase (M_r = 670 000), utilize reduced pyridine nucleotides as the electron donor and have considerably higher molecular weights. In addition, the presence of flavin moieties appears to be associated with the use of reduced pyridine nucleotides as electron donors.

The nitrite reductases have higher specific activities, and correspondingly, larger turnover numbers than the sulfite reductases (Table IV). The larger turnover number for *N. crassa* nitrite reductase (7800 min^{-1}) and spinach nitrite reductase (6588 min^{-1}), may be indicative of the organisms' relative needs for reduced nitrogen versus reduced sulfur.

The striking similarities in the spectral properties, including spectral perturbations caused by inhibitors, the Soret to α ratio, and the absorption maxima of some sulfite and nitrite reductases are due to the presence of a common heme chromophore. That is, siroheme has been found in the *N. crassa* and spinach nitrite reductases, as well as the sulfite reductases from *E. coli* and other bacteria. It remains to be established whether siroheme is also present in *Chlorella* and spinach sulfite reductases. While siroheme has now been identified in the above-mentioned assimilatory nitrite reductases, it is not known if all enzymes capable of reducing nitrite to ammonia contain this prosthetic group. In fact, an enzyme from *Achromobacter fischeri* reported by Prakash and Sadana [33] to catalyze the reduction of nitrite to ammonia presumably as a part of the process of nitrate respiration has heme *c* as its only iron-containing prosthetic group. However, it is important to note that this nitrite reductase is induced only under unphysiological conditions of low oxygen tension. Nitrate respiratory enzymes which catalyze the reduction of nitrite to gaseous products (NO, N_2O, N_2, etc.) do not appear to contain siroheme. Thus, there exists this chemical basis for the physiological distinction between the biological processes of assimilatory and respiratory nitrite reduction in nature.

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