Neurospora crassa NAD(P)H-Nitrite Reductase

STUDIES ON ITS COMPOSITION AND STRUCTURE

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Neurospora crassa nitrite reductase \((M_r = 290,000)\) catalyzes the NAD(P)H-dependent 6-electron reduction of nitrite to ammonia via flavin and siroheme prosthetic groups. Homogeneous \(N.\) crassa nitrite reductase has been prepared employing conventional purification methods followed by affinity chromatography on blue dextran-Sepharose 4B.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of homogeneous nitrite reductase reveals a single subunit band of \(M_r = 140,000\). Isoelectric focusing of dissociated enzyme followed by sodium dodecyl sulfate-gel electrophoresis in the second dimension yields a single subunit spot with an isoelectric point at pH 6.8-6.9. Two-dimensional thin layer chromatography of acid-hydrolyzed nitrite reductase treated with 5-dimethylaminonaphthalene-1-sulfonyl chloride yields a single reactive NH$_2$-terminal corresponding to glycine.

We conclude that \(N.\) crassa nitrite reductase is a homodimer of large molecular weight subunits housing a single subunit spot with an isoelectric point at pH 6.8-6.9. Two-dimensional thin layer chromatography of acid-hydrolyzed nitrite reductase treated with 5-di- methylaminonaphthalene-1-sulfonyl chloride yields a single reactive NH$_2$-terminal corresponding to glycine.

An investigation of the prosthetic groups of nitrite reductase reveals little or no flavin associated with the purified protein, although exogenously added FAD is required for activity in vitro. An iron content of 9-10 Fe eq/mol suggests the presence of nonheme iron in addition to the siohrome moieties. Amino acid analysis yields 43 cysteinyl residues and sulfhydryl reagents react with 50 thiol eq/mol of nitrite reductase. The non-cysteinyl sulfur content, determined as 5.1 acid-labile sulfide eq/mol, is presumably associated with nonheme iron to form iron-sulfur centers.

We conclude that \(N.\) crassa nitrite reductase is a homodimer of large molecular weight subunits housing an electron transfer complex of FAD, iron-sulfur centers, and siohrome to mediate the reduced pyridine nucleotide-dependent reduction of nitrite to ammonia.

Most higher plants, fungi, and bacteria assimilate nitrate by a process which requires the sequential action of two enzymes, nitrate reductase and nitrite reductase. The reduction of nitrate to ammonia involves the transfer of 8 electrons and may be illustrated as follows:

\[
\begin{align*}
\text{NO}_2^- \xrightarrow{\text{nitrate reductase}} \text{NO}_3^- \xrightarrow{\text{2e}^-} \text{NH}_3 \xrightarrow{\text{nitrite reductase}} \text{NH}_4^+.
\end{align*}
\]

Assimilatory nitrate reductases from many bacterial, fungal, and plant sources have been identified and characterized (1). The prokaryotic and plant nitrate reductases utilize NADH as electron donor (2-5), while the enzymes from the fungi \(Aspergillus\) nidulans and \(Neurospora\) crassa use NADPH (6, 7). \(N.\) crassa nitrite reductase \((M_r = 228,000)\) is a soluble, sulfhydryl-containing metalloflavoprotein which mediates the 2-electron transfer sequence (9, and references therein):

\[
\text{NADPH} \rightarrow [\text{SH} \rightarrow \text{FAD} \rightarrow \text{cytochrome b}_{562} \rightarrow \text{Mol} \rightarrow \text{NO}_2^-].
\]

The second enzyme of the nitrate assimilation pathway, nitrite reductase, mediates the 6-electron reduction of nitrite to ammonia. Bacterial nitrite reductases are FAD-dependent metalloproteins and typically utilize NADH as electron donor (10, 11). The enzyme from photosynthetic organisms is characterized as a low molecular weight (60,000-70,000) FAD-independent metalloprotein for which reduced ferredoxin serves as electron donor (12). Nitrite reductases from the fungi \(A.\) nidulans (13) and \(N.\) crassa (14) can accept electrons from either NADH or NADPH. The nitrite reductase complex from \(N.\) crassa is a large molecular weight (290,000) electron-transport system which requires FAD for activity in vitro and which catalyzes the stoichiometric reduction of nitrite to ammonia using 3 eq of reduced pyridine nucleotide to provide the necessary 6 electrons (15).

\(N.\) crassa nitrite reductase was first described by Nason et al. (14). In 1974, Lafferty and Garrett (15) reported a 90-fold purification of \(N.\) crassa nitrite reductase. In addition to the physiological activity of catalyzing the NAD(P)H-dependent reduction of nitrite to ammonia the enzyme also demonstrated FAD-dependent NAD(P)H-hydroxylamine reductase activity as well as FAD-independent dithionite-nitrite reductase activity. In 1975, Vega and Garrett (16, 17) reported that nitrite reductase has an associated NAD(P)H-diaphorase activity for which a dye such as cytochrome c, ferricyanide, menadione, or dichlorodiphenol may serve as electron acceptor. The absorption spectrum of the partially purified nitrite reductase exhibited maxima at 405, 555, 585, and 600 nm (15) and suggested the presence of a novel heme prosthetic group, siroheme. Vega et al. (18) demonstrated, by spectral analysis of \(N.\) crassa nitrite reductase and its extracted heme chromophore, that the heme moiety was indeed siroheme. The oxidized nitrite reductase showed a characteristic heme absorption maximum at 578 nm. A typical "reduced enzyme" spectrum resulted upon the addition of either NADPH and FAD or dithionite. Further spectral changes which represented an interaction of nitrite with the heme moiety resulted when nitrate was added to the reduced enzyme, thereby implying a functional role of the chromophore in nitrite reduction. In addition, incubation of nitrite reductase with CO in the presence of both NADPH and FAD resulted in inhibition of enzymatic activity and the formation of a spectrally distinct complex. Nitrite competed effectively with CO and reversed the inhibition, and the resulting spectrum was identical with the reduced enzyme-nitrite complex spectrum. FAD apparently functions in the transfer of electrons from NAD(P)H to siroheme. Siroheme is presumably the site of nitrite binding. All of the NAD(P)H-dependent activities of nitrite reductase...
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**Experimental Procedures**

**Growth of Mycelia**

A nitrate reductase-deficient strain of *N. crassa*, nit-1, allele 34547 (Fungal Genetics Stock Center No. 54), was grown as described previously (18).

**Purification of Nitrite Reductase**

NAD(P)H-nitrite reductase was purified from *N. crassa* mutant nit-1 (through Fraction 6 of the procedure of Greenbaum et al. (20)). In a typical preparation, 600 g of frozen nit-1 mycelia were homogenized at 0 °C with 1500 ml of 0.1 M potassium phosphate buffer, pH 7.3, containing 5 mM EDTA, 5 mM cysteine, and 10% glycerol. The homogenate was centrifuged at 27,000 × g for 20 min. The resulting supernatant, Fraction 1, was treated by conventional methods of ammonium sulfate fractionation and DEAE-cellulose ion exchange chromatography through the step yielding Fraction 6 (37.5-47.5% (NH₄)₂SO₄ precipitate), which represented a 55% recovery and a 14-fold purification. At this stage of purification, Fraction 6 was divided into 0.3-ml aliquots and stored at −70 °C. To obtain homogeneous nitrite reductase, a single aliquot of Fraction 6 was chromatographed on a Sepharose 4B column (1.5 × 30 cm; bed volume, 50 ml) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 5 mM EDTA, 5 mM cysteine, 10% glycerol, and 2 mM sodium sulfite. Following elution with this buffer, the active fractions were pooled and designated as Fraction 7.

Fraction 7 was immediately chromatographed on a blue dextran-Sepharose 4B affinity column (1.5 × 2.2 cm; bed volume, 4 ml) equilibrated with the previous buffer. The blue dextran-Sepharose 4B resin was prepared according to Ryan and Vestling (21). The column was washed sequentially with 30 mM potassium phosphate equilibration buffer, buffer containing 0.5 mM ATP and 0.5 mM NADP, and buffer alone. Nitrite reductase was eluted from the column with 15 ml of equilibration buffer containing 0.5 mM NADPH to yield the final fraction, the pooled blue dextran-Sepharose 4B eluates (Fraction 8). Recoveries of 30-50% of Fraction 7 and a 1400-fold purification were typical.

**Assay of NADPH-Nitrite Reductase**

Nitrite-dependent pyridine nucleotide oxidation was followed at 340 nm as described previously (15) on either a Cary Model 14 spectrophotometer or a Gilford Model 2400 spectrophotometer. A 1.0-ml reaction mixture included 0.1 ml of 1.0 M potassium phosphate buffer (pH 7.3), 0.1 ml of 10 mM sodium nitrite, 0.1 ml of 0.1 M FAD, and sufficient enzyme to catalyze a decrease in absorbance of 0.005-0.02/min. To initiate the reaction, 0.05 ml of 2 mM NADPH was added.

The N. crassa nitrite reductase was purified to homogeneity by Greenbaum et al. in 1978 (20). The marked instability of previous preparations was overcome and homogeneous enzyme was obtained following affinity chromatography on blue dextran-Sepharose 4B.

The present paper reports the results of physical and chemical studies on the purified N. crassa nitrite reductase. The amino acid composition and subunit organization of the enzyme have been examined. Determinations of the number of various prothigm groups essential to nitrite reductase activity are reported and comparisons to other multielectron transferring systems are drawn.

**Protein Determinations**

Protein concentrations were determined by a modified Lowry method using crystalline bovine serum albumin as the standard (22). In the Fraction 8 samples (blue dextran-Sepharose 4B eluates), the protein was first precipitated by addition of 0.025 ml of 2% sodium deoxycholate and 1 ml of 24% trichloroacetic acid (23). This treatment eliminated interferences caused by the nucleotides and other buffer components. The protein concentration was then determined by the modified Lowry method (22).

**Electrophoretic Analyses**

Polyacrylamide gel electrophoresis was performed according to Clark (24). A discontinuous Tris/glycine buffer system (pH 8.6) was used. Fraction 8 eluates (10-100 μg of protein/0.1- to 0.2-ml samples) were electrophoresed on 5% cross-linked gels for 4 h at 4 °C and 2 mA/tube. SDS-polyacrylamide gel electrophoresis was performed according to the method of Maizel (25). Fraction 8 eluates were dialyzed overnight against 10 mM sodium phosphate buffer, pH 7.2, containing 0.1% SDS and then resolved on 5% polyacrylamide gels as described above.

Following electrophoresis, gels were stained for protein with Coomassie brilliant blue R250 (0.25% in 7% acetic acid and 50% methanol) for 4-12 h and then destained in 570 methanol, 7% acetic acid. Polyacrylamide gels were also stained for enzyme activity. NADPH-diaphorase activity was demonstrated on gels incubated in 0.1 mM potassium phosphate buffer, pH 7.5, containing 10 μM FAD and 0.5 mM NADPH and either of the diaphorase acceptors 0.4 mM nitroblue tetrazolium (27) or 0.1 mM dichloroindophenol (28). Dithionite-nitrite reductase activity was demonstrated on gels incubated in 0.1 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM methyl viologen, 5 mM nitrite, and 0.8% dithionite (29).

**Two-dimensional Gel Electrophoresis**

Samples of homogeneous nitrite reductase were subjected to electrophoretic separation in two dimensions. Electrophoresis in the first dimension employed either the isoelectric focusing procedures of O’Farrell (30) and Ames and Nikaido (31) or the nonequilibrium pH gradient electrophoresis method of O’Farrell et al. (32). In the first method, samples of Fraction 8 were concentrated by ultrafiltration and dissociated by treatment with 2% SDS and 2% β-mercaptoethanol at 100 °C for 5 min. Samples were dialyzed with 2 volumes of dilution buffer (9.5 M urea, 2% amphotiles (pH 3–10), 5% β-mercaptoethanol, 8% Nonidet P-40 (35)). Aliquots of 0.05-0.1 ml (25–50 μg of protein) were layered onto gels prepared according to O’Farrell (30) in glass tubing (110 × 2.5 mm). Electrophoresis proceeded at 300 V for 18 h and then at 400 V for 1 h. Gels were removed and equilibrated in SDS-sample buffer (0.05 M Tris-HCl (pH 7.2), 5% β-mercaptoethanol, 2.3% SDS, 10% glycerol) for 30–60 min prior to electrophoresis in the second dimension.

Electrophoresis in the nonequilibrium system (32) proceeds toward the cathode with 0.01 M H₂PO₄, in the upper reservoir and 0.02 M NaOH in the lower reservoir. Since the polarity is reversed, basic proteins enter the gel first and are better resolved in this system than on isoelectric focusing gels (pH 4–7). Fraction 8 samples were dissociated and prepared in sample dilution buffer as described above. Electrophoresis proceeded for 5–6 h at 300 V. Gels were equilibrated for 30–60 min prior to electrophoresis in the second dimension.

Nitrite reductase samples separated by either first dimension procedure were further resolved by SDS-polyacrylamide gel electrophoresis on 5% slab gels (25). Electrophoresis proceeded at 20 mA for 6-8 h. Slab gels (1.5 mm thick) were stained with 0.5% Coomassie blue R250, 45% ethanol, 10% acetic acid for 30 min.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; dansyl, 5-dimethylaminonapthalene-1-sulfonil.
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Amino Acid Analysis

Amino acid analysis was performed on a Beckman automatic amino acid analyzer Model 120 C. Aliquots of Fraction 8 were hydrolyzed under vacuum in 6 N HCl for 24, 48, and 72 h at 105°C. Cysteine was determined as carboxymethyl cysteine following reduction in the presence of β-mercaptoethanol, alkylation of the reduced protein with iodoacetic acid (33), and hydrolysis at 105°C for 18 h. Tryptophan presence of P-mercaptoethanol, alkylation of the reduced protein with H20. A by-product of the dansylation reaction, dansyl sulfonic acid, was estimated spectrophotometrically by the method of Edelhoch (34).

NH2-terminal Analysis

The NH2-termini of nitrite reductase were labeled with 5-dimethylaminonaphthalene-1-sulfonyl chloride by a modification of the method of Gray (35, 36). Aliquots of Fraction 8 eluates in 0.5 M sodium bicarbonate, pH 9.5, were treated with an equal volume of dansyl chloride (28 mg/ml of acetone) for 16–20 h. The labeled protein was hydrolyzed under vacuum in 6 N HCl at 105°C. After 6–18 h the sample was dried and the amino acids were resuspended in deionized H2O. A by-product of the dnasylation reaction, dansyl sulfonic acid, was extracted with toluene. The dansyl amino acids were extracted with ethyl acetate, dried under N2, and resuspended in methanol.

The dansylated amino preparation (approximately 0.3 nmol of nitrite reductase) was applied to a polyamide thin layer chromatography sheet (20 x 20 cm). Two-dimensional chromatography was performed according to Woods and Wang (37). The dansyl amino acids were separated in a first dimension solvent composed of water and 90% formic acid (100:1.5). After 45 min the sheet was dried and then developed in n-heptane/1-butanol/glacial acetic acid (3:13:11) for 60 min. The fluorescent spots corresponding to the dansyl amino acids were visualized under UV light. The two-dimensional migration of the spots was compared to a reference map constructed with standard dansylated amino acids. In addition, bovine pancreatic insulin served as a standard protein. Labeling, hydrolysis and chromatography of the standard preparations proceed as described above for nitrite reductase.

Prosthetic Group Analysis

Flavin.—The flavin content of homogeneous preparations of nitrite reductase was determined fluorometrically by the method of Faeder and Siegel (38). Samples contained 5–20 µg of protein.

Iron.—Following electrophoresis of the native protein the iron content of nitrite reductase was demonstrated in situ on polyacrylamide gels by two staining methods. Gels were incubated in 0.2% dimethoxybenzidine, 5% acetic acid, 0.05% H2O2 for the dark for 30–45 min. Heme iron appeared as a dark brown band following extensive rinsing of the gel in 15% acetic acid (39). Nonheme iron was stained upon incubation of the gel in 0.7% a,a'-dipyridyl and 8% thioglycollic acid for 10–30 min (40).

The total iron content of nitrite reductase was measured on a Perkin-Elmer atomic absorption spectrophotometer (Model 370) equipped with a graphite furnace HGA 2100 attachment. Aliquots of Fraction 8 (60–80 µg/ml) were dialyzed extensively against distilled water. Samples of 20 µl of standard iron and protein preparations were dried at 110°C for 20 s, charred at 1100°C for 60 s, and atomized at 2500°C for 15 s. Absorbance was measured at 248.3 nm with a slit width of 0.2 nm.

Total iron content was also measured colorimetrically by a modification of the method of Fortune and Mellon (41). Fraction 8 eluates (0.14–0.24 nmol of nitrite reductase/0.4 ml of elution buffer prepared without EDTA) were treated with 0.05 ml of 10% HCl and then incubated at 80°C for 10 min. In rapid succession, 0.25 ml of 10% hydroxylamine and 0.3 ml of 0.5% o-phenanthroline were added. Color development was complete in 15 min and absorbance was measured at 512 nm.

Labile Sulfide Content.—The acid-labile sulfide content of nitrite reductase was determined by measuring methylene blue formation at 670 nm using the procedure described by King and Morris (49) and Siegel et al. (43). Aliquots of 0.15 µg of protein were incubated with the reagent in the presence and absence of 8 µl urea. The absorbance of 4-thiopyridone, the species formed upon reaction of thiols with 4,4'-dithiodipyridine, was measured at 324 nm.

The total sulfhydryl content of nitrite reductase was also determined with 5,5'-dithiobis(2-nitrobenzoic acid) according to the method of Habeeb (45). Fraction 8 eluates were prepared as described above. Samples of native and denatured (+8 M urea) nitrite reductase (23–37 µg of protein) were incubated for 30 min with 2 mm 5,5'-dithiobis(2-nitrobenzoic acid). The absorbance of 2-nitro-5-thiobenzoate was measured at 412 nm.

Materials

NADPH, NADH, NADP, FAD, FMN, blue dextran, Sepharose 4B, RNA polymerase, phosphorylase a, 4,4'-dithiodipiridine, 5,5'-dithiobis(2-nitrobenzoic acid), 5-dimethylaminonaphthalene-1-sulfonyl chloride, and standard dansyl amino acids were purchased from Sigma; ultrapure guanidine HCl and urea were from Schwarz/Mann; all materials for the preparation of polyacrylamide gels were from Bio-Rad; β-galactosidase was from Worthington Biochemicals; and rabbit muscle was aldolase from Calbiochem. Nitrogenase was a gift from Dr. Barbara Burgess. All other chemicals were reagent grade and used as supplied.

RESULTS

Subunit Composition of Nitrite Reductase

The homogeneity of nitrite reductase preparations was routinely confirmed by electrophoresis of blue dextran-Sepharose 4B eluates (Fraction 8) on polyacrylamide gels. Fig. 1A shows the typical single diffuse band corresponding to nitrite reduc–

![Fig. 1. Polyacrylamide gel electrophoresis of purified nitrite reductase. Polyelectrophoresis gels (5%) were prepared as described by Clark (Gel A (24)) and Maizel (Gels B and C (25)). Bromphenol blue, 0.1% w/v, in 10% sucrose was used as a tracking dye. Electrophoresis proceeded for 4 h at 2 mA/tube. Gels were stained for protein with 0.25% Coomassie blue R250 in 7% acetic acid and 50% methanol. Gel A shows the native nitrite reductase (Fraction 8, 30 µg of protein). For Gel B, an aliquot of Fraction 8 containing 25 µg of protein was subjected by treatment with 2% SDS and 2% β-mercaptoethanol. The single protein band corresponds to the nitrite reductase subunit. A single band is also shown on Gel C for which an aliquot of Fraction 8 was denatured in the presence of 8 M guanidine HCl and 50 mM β-mercaptoethanol.](fig1.png)
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Two-dimensional Electrophoresis

Isoelectric focusing of dissociated nitrite reductase samples in a pH gradient of pH 4-7 or electrophoresis in a nonequilibrium pH gradient followed by a size separation of subunits on a 5% slab gel in a Tris/glycine/0.1% SDS system in the second dimension revealed a single protein spot corresponding to the M₀ = 140,000 subunit of nitrite reductase. The subunit has an isoelectric point of pH 6.8-6.9. The homodimeric nature of the enzyme is further supported since the subunits demonstrated the same isoelectric point as well as identical molecular weight.

Amino Acid Composition

The amino acid composition of nitrite reductase is shown in Table I. A partial specific volume of 0.730 cm³ g⁻¹ was calculated by the method of Cohn and Edsall (46). Assuming integral values for the amino acid composition, a molecular weight of 289,353 was calculated.

NH₂-Terminal Analysis

The NH₂-termini of nitrite reductase were labeled with 5-dimethylaminonaphthalene-1-sulfonamide chloride. Following hydrolysis of the protein and partial removal of the by-products of the dansylation procedure, the highly fluorescent, ethyl acetate-extracted dansyl amino acids were applied to a polyamide thin layer chromatography sheet. Fig. 3 shows a UV-

Table I: Amino acid composition of nitrite reductase

| Amino acid      | Residues/M₀ | % total residues |
|-----------------|-------------|-----------------|
| Alanine         | 202         | 7.6             |
| Arginine        | 131         | 4.9             |
| Aspartic acid and asparagine | 265 | 10.0 |
| Cysteine⁴      | 43          | 1.6             |
| Glutamic acid and glutamine | 268 | 10.0 |
| Glycine         | 292         | 11.0            |
| Histidine       | 48          | 1.8             |
| Isoleucine      | 108         | 4.1             |
| Leucine         | 233         | 8.8             |
| Lysine          | 175         | 6.6             |
| Methionine      | 38          | 1.4             |
| Phenylalanine   | 81          | 3.0             |
| Proline         | 130         | 4.9             |
| Serine⁶         | 192         | 7.2             |
| Threonine⁷      | 158         | 6.0             |
| Tryptophan⁸     | 78          | 2.9             |
| Tyrosine        | 53          | 2.0             |
| Valine          | 158         | 6.0             |
| Basic           |             | 10.0            |
| Aromatic        |             | 7.9             |
| Hydrophobic     |             | 37.8            |

tion on a 5% gel stained with Coomassie brilliant blue R250.

Samples of homogeneous nitrite reductase were dissociated with SDS and β-mercaptoethanol and electrophoresis in a discontinuous Tris/glycine/0.1% SDS buffer system followed. Fig. 1B shows the single protein band observed following such treatment on a 5% polyacrylamide gel. Similarly, samples of nitrite reductase denatured in the presence of guanidine hydrochloride revealed one sharp protein band following electrophoresis (Fig. 1C).

The molecular weight of the nitrite reductase subunit corresponding to the single protein band was determined by co-electrophoresis of SDS-dissociated nitrite reductase and standard proteins of known subunit molecular weight. A plot of mobility factor versus log of the subunit molecular weight revealed a subunit molecular weight of 140,000 for nitrite reductase (Fig. 2). N. crassa nitrite reductase (M₀ = 290,000) is apparently a dimer of identical molecular weight subunits.

Fig. 2. Nitrite reductase subunit molecular weight. SDS-polyacrylamide gels (5%) were prepared according to Maizel (25). Samples of homogeneous nitrite reductase (Fraction 8, 10-50 µg of protein), dissociated by treatment with 2% SDS and 2% β-mercaptoethanol, were electrophoresed with myosin, RNA polymerase, and phosphorylase. Electrophoresis and staining of gels proceeded as described under "Experimental Procedures."

Fig. 3. Two-dimensional thin layer chromatography of dansylated nitrite reductase. Fraction 8 samples in 0.5 M NaHCO₃ buffer, pH 9.5 (80 µg/0.2 ml), were incubated for 20 h with 0.2 ml of dansyl chloride (20 mg/ml of acetone) and then hydrolyzed for 18 h at 105 °C in 6 N HCl (35, 36). The dansylated NH₂-terminal amino acid (spot C) was partially separated from the by-products of the procedure, dansyl sulfonic acid (A) and dansyl sulfonamide (B), by a series of toluene and ethyl acetate extractions. Labeled amino acids were applied to a polyamide sheet (20 x 20 cm) which was developed in the first dimension in H₂O/90% formic acid (100:1.5) for 45 min. The sheet was air-dried and developed in the second dimension in n-heptane/1-butanol/glacial acetic acid (3:3:1) for 60 min. The fluorescent spots were visualized under UV light.

The illuminated polyamide sheet on which a dansylated nitrite reductase preparation was chromatographed in two dimensions: the single fluorescent amino acid spot (C) was distinguished from the by-products, dansyl sulfonic acid (A) and dansyl sulfonamide (B) by the color of the fluorescence. Dansyl sulfonic acid demonstrated a blue fluorescence, dansyl sulfonamide appeared orange and the dansyl amino acid was yellow when visualized under UV light.
The dansylated nitrite reductase preparation was also chromatographed in a mixture of several standard dansyl amino acids. When compared to a two-dimensional map constructed with 18 dansyl amino acids, the single fluorescent product of the reaction of nitrite reductase with dansyl chloride corresponded to dansyl glycine.

Prosthetic Group Analysis

Flavin—Quantitative fluorescent measurements of the flavin content of homogeneous nitrite reductase were made by a procedure based on the pH-dependent fluorescent behavior of FAD and FMN. The fluorescence of supernatants from boiled Fraction 8 eluates was measured at pH 7.7 and pH 2.6. Table II shows that very little flavin is associated with the purified protein. The amount of FAD ranged from 0.13-0.2 mol/mol of nitrite reductase.

Iron Analysis—Atomic absorption spectroscopy measurements of the iron content of homogeneous nitrite reductase preparations dialyzed extensively against glass-distilled water gave an average value of 10.2 mol of Fe/mol of nitrite reductase (Table II). An alternate analytical procedure, based on the reaction of reduced iron with o-phenanthroline, permitted the direct assay of iron in Fraction 8 eluates. An average value of 3 mol of iron/mol of nitrite reductase was obtained by this method (Table II). The total iron content of nitrite reductase presumably includes heme iron associated with siroheme moieties and nonheme iron involved in iron-sulfur centers.

Sulfhydryl Analysis—The available versus total sulfhydryl content of nitrite reductase was analyzed with the thiol reagent 4,4'-dithiodipyridine. The reaction of standard cysteine solutions with 4,4'-dithiodipyridine was complete in 5 min and absorbance at 324 nm was linear from 2-20 nmol of sulfhydryl equivalents. The standard proteins, Klebsiella nitrogenase and rabbit muscle aldolase, required a 90 min incubation period in the presence of 8 M urea, to completely expose and react all sulfhydryl equivalents. Table III shows that under these reaction conditions, 50 sulfhydryl eq/mol of nitrite reductase reacted with 0.08 mM 4,4'-dithiodipyridine. In the absence of urea, only 37.4 sulfhydryl groups were reactive or accessible.

The sulfhydryl content of homogeneous nitrite reductase was also examined with 5,5'-dithiobis(2-nitrobenzoic acid). A high concentration of reagent and the addition of 8 M urea to incubation mixtures were required for complete reaction of sulfhydryl equivalents. Table III shows that, after 30 min, 50 sulfhydryl eq/mol of nitrite reductase reacted with 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 8 M urea. Only 34.6 sulfhydryl eq/mol of nitrite reductase were accessible in the native enzyme. No additional sulfhydryl groups were demonstrated with 4 mM 5,5'-dithiobis(2-nitrobenzoic acid).

Substrate Affinities

The Michaelis constants of NADPH-nitrite reductase for the substrates NADPH and nitrite and the cofactor FAD were determined with homogeneous enzyme preparations. Nitrite-dependent NADPH oxidation was followed at 340 nm and initial velocities were determined from absorbance values recorded every 3 s over a 20-s period. A least squares regression analysis of all kinetic data was performed. The apparent $K_m$ values are 15 μM for NADPH, 7.5 μM for nitrite, and 0.02 μM for FAD.

DISCUSSION

The Neurospora crassa nitrite reductase ($M_w = 290,000$) has been purified to homogeneity by conventional methods of ammonium sulfate fractionation, DEAE-cellulose ion exchange chromatography, and Sepharose 4B gel filtration chromatography, followed by affinity chromatography on blue dextran-Sepharose 4B. The purified enzyme exhibited one band upon polyacrylamide gel electrophoresis under nondenaturing conditions. Various staining techniques were employed to demonstrate that heme iron, nonheme iron, and two partial activities of $N$. crassa nitrite reductase, namely the NADPH-diaphorase and the dithionite-nitrite reductase activities, were associated with this nitrite reductase protein band.

Electrophoresis of homogeneous nitrite reductase under denaturing conditions revealed a single protein band of $M_w = 140,000$. Denaturation of the enzyme in the presence of 8 M guanidine hydrochloride followed by alkylation and SDS-polyacrylamide gel electrophoresis also resulted in a single protein band, indicating that the smallest dissociable unit of nitrite reductase has a molecular weight of 140,000.

Results of two-dimensional polyacrylamide gel electrophoresis indicate that the subunits are identical in isoelectric point as well as molecular weight. Separation of subunits in either the isoelectric focusing or nonequilibrium pH gradient system yielded a single protein spot on the second dimension SDS-polyacrylamide gel. The $M_w = 140,000$ subunit has an isoelectric point at pH 6.8-6.9.

Two-dimensional thin layer chromatography of dansylated nitrite reductase revealed a single fluorescent-labeled NH$_2$-terminal amino acid corresponding to glycine. Nitrite reductase apparently possesses only one type of reactive NH$_2$ terminus. Since the nitrite reductase subunits also display identical isoelectric points and molecular weights, it is concluded that $N$. crassa nitrite reductase is a homodimeric protein. In addition, a single $N$. crassa locus, the nit-6 gene, has been designated as the structural gene encoding the nitrite reductase apoprotein (47).

Atomic absorption spectroscopy of nitrite reductase purified to homogeneity yielded an average of 10.2 Fe eq/mol of nitrite reductase. Colorimetric determinations of total iron with o-phenanthroline gave an average value of 9 Fe/mol of nitrite reductase. Since it is assumed that each subunit of nitrite reductase binds a single siroheme, presumably 2 iron eq correspond to the heme iron of the siroheme moiety. This hypothesis is supported by the evidence that the sulfite reductases of $E$. coli and spinach (48) bind one siroheme/hemoprotein subunit.

| Table II | Quantitation of prosthetic groups of nitrite reductase |
|-----------------|----------------------------------|
| Component | Equivalents | Method of assay |
| FAD | 0.18 | pH-dependent fluorescence |
| FMN | 0.11 | pH-dependent fluorescence |
| Iron | 10.2 | Atomic absorption spectroscopy |
| Acid-labile sulfide | 8.1 | Dimethylphenylenediamine-methylene blue formation |

*a Measured per molecule of nitrite reductase.*

| Table III | Sulphydryl content of nitrite reductase |
|-----------------|----------------------------------|
| Reagent | Concentration | 8 M urea | -SH eq/mol |
| 4,4'-Dithiodipyridine | 0.08 | 37.4 |
| 5,5'-Dithiobis(2-nitrobenzoic acid) | 0.08 | 50.0 |
| Dimethylphenylenediamine-methylene blue formation | 1.0 | 27.8 |
| Dimethylphenylenediamine-methylene blue formation | 1.0 | 36.2 |
| Dimethylphenylenediamine-methylene blue formation | 2.0 | 34.6 |
| Dimethylphenylenediamine-methylene blue formation | 4.0 | 50.0 |

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Estimation of the acid-labile sulfide content of *N. crassa* nitrite reductase by the methylene blue formation assay shows 8.1 $S^2$ mol of enzyme. Thus the remainder of the iron as determined above is apparently associated with sulfur as nonheme iron-sulfur centers which may be present as four Fe$_4$S$_4^*$ or two Fe$_2$S$_2^*$ centers. It is likely that the iron is organized as tetranuclear centers in nitrite reductase since other 6-electron transferring enzymes such as *E. coli* sulfite reductase (48), spinach nitrite reductase (49), and nitrogeanse (50) contain Fe$_4$S$_4^*$ centers.

The sulphydryl reagents, 5,5'-dithiobis(2-nitrobenzoic acid) and 4,4'-dithiodiiodoppyridine, each reacted with 50 sulhydryl eq/mol of nitrite reductase. Amino acid analysis of carboxymethylated nitrite reductase shows 43 carboxymethylcysteinyl residues. The difference of 7 sulhydryl eq confirms the presence of noncysteine sulfur associated with the nonheme iron-sulfur centers. In addition, titration of total versus available sulphydryl groups indicated that a class of relatively inaccessible or slow reacting sulphydryl equivalents exists. An interesting speculation is that the 14-16 groups which consistently required the addition of 8 mM and high concentrations of reagents for complete reaction may be the sulfide equivalents of two Fe$_4$S$_4^*$ and 8 cysteinyl sulfurs which are necessary to bind the centers to the protein.

In the absence of FAD in highly purified preparations of *N. crassa* nitrite reductase, there are indications that the enzyme exists as a flavoenzyme in vivo. The addition of exogenous FAD is required in vitro for maximal expression of enzymatic activity. Spectral perturbations in the 580-nm absorption band of siroheme upon incubation of nitrite reductase with NADPH $\pm$ FAD suggested that the flavin prosthetic group mediates the transfer of electrons between NADPH and siroheme (18). In addition, the $K_m$ for FAD is 0.2 $\mu$M; the constants for the substrates of the reaction, NADPH and nitrite, are 400- to 800-fold greater.

Comparison of assimilatory nitrite reductase of heterotrophic organisms, where this nitrite reductase from *N. crassa* serves as the prototype, with the nitrite reductase of phototrophic organisms, the spinach enzyme being the best characterized example (49, and references therein), suggests a synthetic organisms, the spinach enzyme being the best characterized example (49, and references therein), suggests a synthetic role of nitrite reduction. Both contain nonheme iron-sulfur centers. Spinach nitrite reductase employs photosynthetically reduced ferredoxin to dramatically in their electron donor specificity. Spinach nitrite reductase with NADPH and siroheme (18). In addition, the flavoenzyme subunit has been examined by spectrophotometric and EPR studies. Rueger and Siegel (53) showed that spectral alterations at siroheme absorption maxima are induced upon reaction of sulfite reductase with CO or CN- or with the substrate sulfite. $\text{S}_2\text{O}_3^{2-}$ was shown to bind and remain closely associated with the heme moiety during the course of its reduction. In addition, Siegel (48) demonstrated that the Fe$_4$S$_4^*$ center undergoes a modification in EPR signal and is rendered more readily reducible upon the formation of CO or CN-siroheme complexes which suggests that there is no interaction between the heme and Fe-S center of sulfite reductase during sulfite turnover. Siroheme has been designated as the nitrite binding site for spinach nitrite reductase and a role for the Fe$_4$S$_4^*$ center in nitrite reduction has been proposed by Lancaster et al. (49). Recently, Christner et al. (54) demonstrated by EPR and Mössbauer spectroscopic analyses that the siroheme and Fe-S center of the *E. coli* sulfite reductase hemoprotein subunit are closely linked as a functional unit. A common bridging ligand may provide the linkage since the two chromophores appeared to share a single electronic spin and the electronic environments of 5 Fe atoms were altered upon the addition of a single electron to the oxidized enzyme.

Since the reduction of sulfite or nitrite involves the transfer of 6 electrons and the maximum electron storage capacity of either *E. coli* sulfite reductase hemoprotein subunit or spinach nitrite reductase is apparently only 2 electrons (1 in the Fe-S center and 1 in siroheme), Siegel (48) concludes that intermediates of the reaction remain bound throughout the catalytic cycle. Indeed, like *E. coli* sulfite reductase, *N. crassa* nitrite reductase can catalyze the 6-electron reduction of nitrite utilizing dithionite as reductant. The iron-sulfur centers and siroheme are probably involved in this reaction in a manner similar to that described for *E. coli* sulfite reductase and spinach nitrite reductase. However, the presence of FAD prosthetic groups as well as sulphydryl groups which may be catalytically active raises the electron storage capacity of *N. crassa* nitrite reductase.
The ability of nitrite reductase to catalyze a 6-electron reaction is one of the most interesting properties of the enzyme. Other proteins catalyzing multielectron transfers are typically heteromultimeric, including cytochrome oxidase, which catalyzes the 4-electron reduction of O₂ to H₂O, and the 6-electron transferring enzymes sulfite reductase and nitroreductase. Cytochrome oxidase, electron transferring enzymes sulfite reductase and nitroreductase have the subunit structure as described (50). The fact that N. crassa nitrite reductase is constituted as a homodimer of high molecular weight subunits which house a complex electron transfer apparatus places this enzyme in a unique position among the proteins which catalyze multielectron transfers.

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REFERENCES

1. Payne, W. J. (1973) Bacteriol. Rev. 37, 410-422
2. Nicholas, D. J. D., and Nason, A. (1955) J. Bacteriol. 68, 580-583
3. Guerrero, M. G., Vega, J. M., Leadbetter, E., and Losada, M. (1973) Arch. Mikrobiol. 91, 307-309
4. Schloemer, R. H., and Garrett, R. H. (1973) Plant Physiol. 51, 561-563
5. Paneque, A., Del Campo, F. F., Ramírez, J. M., and Losada, M. (1965) Biochim. Biophys. Acta 109, 79-85
6. Downey, R. J. (1971) J. Bacteriol. 105, 759-768
7. Nason, A., and Evans, H. J. (1953) J. Biol. Chem. 202, 655-673
8. Garrett, R. H., and Nason, A. (1969) J. Biol. Chem. 244, 2870-2882
9. Garrett, R. H., and Amy, N. K. (1978) Adv. Microb. Physiol. 18, 1-65
10. Vega, J. M., Guerrero, M. G., Leadbetter, E., and Losada, M. (1975) Biochem. J. 133, 701-708
11. Coleman, K. J., Cornish-Bowden, A., and Cole, J. A. (1978) Biochem. J. 175, 483-493
12. Losada, M., and Guerrero, M. G. (1979) in Photosynthesis in Relation to Model Systems (Barber, J., ed) pp. 365-408, Elsevier/North Holland Biomedical Press, Amsterdam
13. Pateman, J. A., Rever, B. M., and Cove, D. J. (1977) Biochem. J. 164, 103-111
14. Nason, A., Abraham, R. G., and Averbach, B. C. (1954) Biochim. Biophys. Acta 15, 159-161
15. Lafferty, M. A., and Garrett, R. H. (1974) J. Biol. Chem. 249, 7595-7597
16. Vega, J. M., and Garrett, R. H. (1975) Proceedings of the sixth Congress of the Spanish Society of Biochemistry, p. 108
17. Vega, J. M. (1976) Arch. Mikrobiol. 109, 237-242
18. Vega, J. M., Garrett, R. H., and Siegel, L. M. (1975) J. Biol. Chem. 250, 7980-7989
19. Garrett, R. H. (1978) in Microbiology (Schlessinger, D., ed) pp. 324-329, American Society for Microbiology, Washington D.C.
20. Greenbaum, P., Prodouz, K. N., and Garrett, R. H. (1978) Biochim. Biophys. Acta 526, 52-64
21. Ryan, L. D., and Vestling, C. S. (1974) Arch. Biochem. Biophys. 160, 279-284
22. Layne, E. (1957) Methods Enzymol. 3, 983-984
23. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241-250
24. Clark, J. T. (1964) Ann. N. Y. Acad. Sci. 121, 428-436
25. Maizel, J. V., Jr. (1971) Methods Virol. 8, 179-246
26. Weber, K., Pringle, J. R., and Osborn, M. (1972) Methods Enzymol. 26, 3-27
27. Cohn, M. L., Wagel, L., Scouwen, W., and McManus, I. R. (1968) Biochim. Biophys. Acta 159, 182-185
28. Brewer, G. W., and Sing, C. F. (1970) An Introduction to Isozyme Techniques, pp. 113-114, Academic Press, New York
29. Hucklesey, D. P., and Hagenman, R. H. (1973) Anal. Biochem. 56, 591-592
30. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007-4021
31. Ames, G. F.-L., and Nikaido, K. (1976) Biochemistry 15, 616-623
32. O’Farrell, P. Z., Goodman, H. M., and O’Farrell, P. H. (1977) Cell 12, 1139-1142
33. Anfinsen, C. B., and Haber, E. (1961) J. Biol. Chem. 236, 1361-1363
34. Edelhoch, H. (1967) Biochemistry 6, 1948-1954
35. Gray, W. R. (1967) Methods Enzymol. 11, 139-151
36. Gray, W. R. (1972) Methods Enzymol. 15, 121-138
37. Woods, K. R., and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369-370
38. Faeder, E. J., and Siegel, I. M. (1973) Anal. Biochem. 53, 322-336
39. E-C Apparatus Corporation Technical Bulletins #141 and #145, Philadelphia
40. Brill, W. J., Westphal, J., Steighorst, M., Davis, L. C., and Shah, V. K. (1974) Anal. Biochem. 60, 297-304
41. Fortune, W. B., and Mellon, M. G. (1938) Ind. Engng. Chem. Anal. Edn. 10, 60-64
42. King, T. E., and Morris, R. O. (1967) Methods Enzymol. 10, 634-641
43. Siegel, L. M., Murphy, M. J., and Kamin, H. (1973) J. Biol. Chem. 248, 251-264
44. Grassetti, D. R., and Murray, J. F., Jr. (1967) Arch. Biochem. Biophys. 119, 41-49
45. Habeeb, A. F. A. (1972) Methods Enzymol. 25, 457-464
46. Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino acids, and Peptides, p. 374, Reinhold Publishing Corp., New York
47. Tomsett, A. B., and Garrett, R. H. (1980) Genetics 95, 649-660
48. Siegel, L. M. (1978) in Mechanisms of Oxidizing Enzymes (Singer, T. P., ed) pp. 201-214, Elsevier/North Holland, Inc., Amsterdam
49. Lancaster, J. R., Vega, J. M., Kamin, H., Orme-Johnson, N. R., Orme-Johnson, W. H., Krueger, R. J., and Siegel, L. M. (1979) J. Biol. Chem. 254, 1268-1272
50. Mortenson, L. E., and Thorneley, R. N. F. (1979) Annu. Rev. Biochem. 48, 387-418
51. Siegel, L. M., and Davis, P. S. (1974) J. Biol. Chem. 249, 1587-1598
52. Amy, N. K., Garrett, R. H., and Anderson, B. M. (1977) Biochim. Biophys. Acta 480, 83-95
53. Rueger, D. C., and Siegel, L. M. (1976) in Flavins and Flavoproteins (Singer, T. P., ed) pp. 610-620, University Park Press, Baltimore
54. Christner, J. A., Munch, E., Janick, P. A., and Siegel, L. M. (1981) J. Biol. Chem. 256, 2098-2101
55. Fry, M., Yende, H., and Green, D. E. (1978) Proc Natl. Acad. Sci. U. S. A. 75, 5908-5911