Neurospora crassa NAD(P)H-nitrite reductase, encoded by the nit-6 gene, is a soluble, α2-type homodimeric protein composed of 127-kDa polypeptide subunits. This multicenter oxidation-reduction enzyme utilizes either NADH or NADPH as electron donor and possesses as prosthetic groups two iron-sulfur (Fe3S4) clusters, two siroheme groups, and two FAD molecules. The native activity of the enzyme is the NAD(P)H-dependent reduction of nitrite to ammonia. In addition, N. crassa nitrite reductase displays several partial activities in vitro, including a siroheme-independent NAD(P)H-cytochrome c reductase activity and an FAD-independent dithionite-nitrite reductase activity. These partial activities are presumed to be manifestations of discrete functional domains within the protein. A full-length nit-6 cDNA was constructed and used in developing an expression system within E. coli capable of yielding high levels of NADPH-nitrite reductase activity. Maximal expression was obtained in E. coli cells grown anaerobically at 22 ± 1 °C, in conjunction with co-expression of a plasmid-borne casG gene (encoding the rate-limiting enzyme in siroheme synthesis) and co-transformation with plasmid pGroESL (encoding bacterial chaperonins GroES and GroEL). Dissection of gene segments encoding putative functional domains within the nit-6 gene was performed. Expression of a partial cDNA construct encoding the FAD/NAD-binding domain yielded extracts with NADPH-cytochrome c reductase activity but no NADPH-nitrite reductase activity or dithionite-nitrite reductase activity. Expression of a cDNA construct encoding the (Fe3S4)-siroheme-binding domain resulted in extracts possessing dithionite-nitrite reductase activity but no NADPH-nitrite reductase or NADPH-cytochrome c reductase activity. Analysis of site-directed mutations altering amino acid residues Cys-331 within the FAD/NAD-binding domain and Ser-755 within the (Fe3S4)-siroheme-binding domain of the nitrite reductase demonstrated that these residues were not essential for native or partial enzyme activity. Cys-757 within the (Fe3S4)-siroheme-binding domain was essential for native enzyme activity.

Nitrate assimilation is a two-step enzymatic process: the reduction of nitrate to nitrite, a two-electron transfer reaction catalyzed by the enzyme nitrite reductase, followed by the reduction of nitrite to ammonium, a six-electron transfer reaction catalyzed by the enzyme nitrite reductase (1–4). In the filamentous fungus Neurospora crassa, NAD(P)H-nitrite reductase (EC 1.6.6.4) is an α2-type homodimer of Mr 290,000, which utilizes either NADPH or NADH as electron donor and possesses two iron-sulfur (Fe3S4) clusters, two siroheme cofactor groups, and two FAD molecules (5, 6). The enzyme requires three equivalents of NADH or NADPH to pass six electrons to the substrate nitrite, converting it to ammonia. Besides this native enzyme activity, N. crassa nitrite reductase catalyzes in vitro partial activities, which include an FAD-dependent NADPH-cytochrome c reductase activity, and dithionite (S2O42−)-nitrite reductase activity (5–8). The NAD(P)H-cytochrome c reductase activity appears to short cut the (Fe3S4)-siroheme-containing portion of the enzyme, while the dithionite-nitrite reductase activity bypasses the requirement for NADPH and FAD. The electron flow within the enzyme complex has been deduced primarily through inhibition studies on the various activities of the enzyme (5–7) and is illustrated in the following scheme where brackets define the enzyme complex and (-SH) represents a sulphydryl moiety presumed to be involved in early electron transfer reactions (6).

Dithionite (S2O42−) → 

\[ 3 \text{NADPH} \rightarrow [(\text{-SH}), \text{FAD} \rightarrow \text{Fe}3\text{S}4, \text{Siroheme}] \rightarrow \text{NO}2^- \] 

Cytochrome c

Scheme 1

Deduced amino acid sequence data suggest that functional domains of fungal nitrite reductases as well as Escherichia coli NADH-nitrite reductase are laid out in a linear fashion along the polypeptide chain, beginning with the FAD- and NADPH-dependent functions at the N-terminal end and ending with the nitrite-reducing functions at the C terminus (9). Similarly, the functional domains of nitrate reductases from fungi and plants are laid down in a linear fashion along the protein, but in reverse order (9). In the case of nitrate reductases, the domains are well defined. Hinge regions bridge the domains within the protein, as supported by the fact that proteolytic degradation of nitrate reductases yields polypeptide fragments with partial enzyme activities (2, 9). In contrast, the domain structures of nitrite reductase are not well defined, and no hinge regions bridging putative domains have been identified through proteolysis (2, 9).

Exley et al. (10) previously reported the nucleotide sequence of the nit-6 gene and the deduced amino acid sequence of the nit-6 protein (10). In that study, putative functional domains and amino acid residues within the N. crassa NAD(P)H-nitrite

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed. Tel.: 804-982-5494; Fax: 804-982-5626.
tions of the amino acid side chain at this position led Exley and coworkers (9) to propose that a Cys residue at position 331 of the polypeptide (10). A conserved feature found within the FAD-/NAD-binding domain is a Cys residue at position 331 of the polypeptide (10).

Other enzymes utilizing an (Fe₅S₅)-siroheme cluster, including nitrite reductase from spinach (16), maize (17), and birch (18) reductase, might be important to the functional integrity of the (Fe₅S₅)-siroheme cluster. In this study, an expression system in E. coli capable of yielding high levels of functional NAD(P)H-nitrite reductase is described. The results of a dissection of nit-6 gene segments encoding functional domains within N. crassa nitrite reductase are reported. Effects on native and partial enzyme activities due to the alteration of amino acid residues Cys-331, Ser-755, and/or Cys-757 through site-directed mutagenesis are also presented.

MATERIALS AND METHODS

Neurospora Strains and Culture Conditions—N. crassa wild-type strain 74-OR23-1 (stock number 987, Fungal Genetics Stock Center, Department of Microbiology, University of Kansas, Kansas City, KS) was utilized in preparing mycelial cell-free extracts for controls in nitrite reductase enzymatic assays and immunoblotting studies. Cultures of mycelia were grown aerobically in 1 L of Vogel’s medium (20) plus 3% sucrose at 30 °C, with the modification that NH₄NO₃ was omitted. Instead, different nitrogen sources were added to the medium depending on the type of nitrogen nutrition desired. Mycelia routinely were grown for 18–24 h from conidial inocula in medium containing 20 mM glutamine and then harvested by filtration, washed with distilled water, and transferred into media containing either glutamine or sodium nitrate as the sole nitrogen source. Mycelia were cultured for 6 h in media containing 20 mM NaN₃ to induce nitrate assimilation or 20 mM glutamine to repress it. Harvested mycelia were washed in distilled water, frozen in liquid nitrogen and stored at −80 °C.

Preparing Cell-free Extracts of N. crassa—Frozen pads of mycelia were homogenized on ice in 7 ml Ten Broeck homogenizers in approximately 4 volumes of ice-cold extraction buffer (20 mM KPO₄, pH 7.5, 10% glycerol, 5 mM EDTA, and 10 μM FAD; just before use, fresh dry cysteine was added to a final concentration of 5 mM, and dry phenylmethylsulfonyl fluoride was added to a final concentration of 2 mg/ml). The homogenates were immediately centrifuged at 20,000 × g for 30 min at 0–4 °C. Supernatants were decanted into fresh tubes and assayed for NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase activity.

E. coli Strains and Culture Conditions Used for Protein Expression—E. coli strains and protein expression vectors used in this study are shown in Table I. E. coli colonies grown with appropriate antibiotic selection were picked from Luria broth (LB) plates and used to inoculate overnight cultures grown at 37 °C in LB containing the appropriate antibiotic. These cultures were grown to stationary phase and used to inoculate the liquid medium sustaining growth during heterologous protein expression (by inoculating fresh medium with 1/250 volume of overnight cultures). Minimal medium was prepared by making a 20 × monobasic potassium phosphate, and 2 g of ammonium sulfate per liter salt solution containing 210 g of dibasic potassium phosphate, 90 g of monobasic potassium phosphate, and 20 g of ammonium sulfate per liter. This solution was diluted to give a final volume of 1 × after adding 0.25 ml of trace metal solution (20) per liter and then sterilized. After cooling, the solution received 25 ml of a sterile 20% solution of glucose per liter (final volume) and 2 ml of 10× magnesium sulfate per liter (final volume). The appropriate antibiotics were then added, followed by inoculation with the stationary culture.

Growth of E. coli—Aerobic E. coli cultures were grown in 500 ml to 1 liter of medium in 2-liter shaker flasks at 250 rpm in a New Brunswick series 25 shaker incubator. Room temperature during aerobic growth was consistently 27 ± 1 °C. Aerobic cultures were induced for protein expression when the cultures reached an A₅₅₀ of 0.3–0.5 by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM. For anaerobic growth, E. coli cells were propagated in large culture volumes of 8–10 liters in a New Brunswick Microform fermentor. The air intake valve of the fermentor was attached to a tank of pure nitrogen. Medium was flushed with N₂ for 1 min while mixing at 300 rpm, both prior to and after inoculating the media with starter culture and after adding IPTG. Room temperature during anaerobic growth was consistently at 22 ± 1 °C. Induction of anaerobic cultures was carried out when the cultures reached an A₅₅₀ of 0.1–0.2 (typically after 24 h of growth on minimal medium at room temperature) by adding IPTG to a final concentration of 0.8 mM. Maximal levels of NADPH-nitrite reductase were found in cells grown anaerobically in minimal medium, 15 h following IPTG induction. Therefore, these conditions were used for routine protein expression.

Preparing Cell-free Extracts of E. coli—E. coli cultures grown under...
either aerobic or anaerobic conditions were harvested by centrifugation at 4500 \( \times g \) for 15 min. The cell pellets were resuspended and washed in 0.5 times the original culture volume with 0.9% NaCl. The cells were then collected by centrifugation, and the cell pellets were stored at \(-70^\circ C\). Frozen cell paste was thawed on ice, mixed with approximately 4–10 volumes of ice-cold extraction buffer, and sonicated at 70 mA for 50 s (Branson sonifier, Branson Sonic Power Co.) in ice-jacketed 50-ml stainless steel beakers. Cell debris was removed by centrifugation at 30,000 \( \times g \) for 30 min. The supernatants were decanted into fresh tubes on ice and immediately assayed for NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase activities.

**Enzyme Assays**—NADPH-nitrite reductase activity was measured spectrophotometrically by following the nitrite-dependent oxidation of nitroblue tetrazolium. Cell debris was removed by centrifugation at 30,000 \( \times g \) for 30 min. The supernatants were decanted into fresh tubes on ice and immediately assayed for NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase activities.

**Protein Determination**—The protein concentration of each cell-free extract was determined using the Protein Dye Concentrate system from Bio-Rad.

**DNA Isolation, Purification, and Subcloning**—Plasmid DNA was propagated in *E. coli* strain DH5\( _{a} \) or JM109 with the appropriate antibiotic selection overnight. Plasmid DNA was isolated and purified as described by Sambrook, Fritsch, and Maniatis (21) or by using DNA purification columns (Qiagen). DNA restriction fragments were purified from low melt gels using the GeneClean kit from Bio 101. Standard techniques were used in DNA subcloning (21).

**PCR-Rapid Amplification of cDNA Ends and Full-length nit-6 cDNA Construction**—PCR-rapid amplification of cDNA ends (22) was used in constructing a full-length nit-6 cDNA (Fig. 1). Poly(A)\(^{+}\) RNA was isolated from nitrate-induced *N. crassa* wild-type mycelia as described previously (10). Three separate reverse transcription reactions were performed using three different oligomer primers designed to hybridize to nit-6 mRNA in the poly(A)\(^{+}\) RNA fraction. One \( \mu g \) of poly(A)\(^{+}\) RNA was combined with 10 pmol of primer (16 nucleotides in length) designed to hybridize downstream of introns 1 and 2, intron 3, and at the 3′ end of the nit-6 transcript. Reverse transcription reactions were performed as recommended by the manufacturer (Life Technologies, Inc.). Upon completion of the reaction, 2 units of RNase H and 9 units of RNase \( T_{1} \) were added, and the mixture was incubated at 37 °C for 15 min. This reaction mixture was extracted with phenol/chloroform, and the DNA was ethanol-precipitated. The DNA was then dissolved in 500 \( \mu l \) of TE (10 mM Tris-Cl, pH 8, 1 mM EDTA). A 10-\( \mu l \) aliquot from each first-strand cDNA reaction was used as template for PCR to produce and amplify double-stranded DNA.

Oligonucleotide primers were designed to hybridize adjacent to existing restriction sites in order to facilitate ligations and cloning (Fig. 1). Primers hybridizing to the 5′ and 3′ ends of the nit-6 cDNA were designed to introduce restriction sites (NdeI and BamHI) for subcloning into the expression vector pET11a. Each PCR reaction contained a 10-\( \mu l \) aliquot of first-strand cDNA diluted in TE, 60 pmol of primer, 100 \( \mu l \) of dNTPs, in a total volume of 100 \( \mu l \) of 1× *Pfu* polymerase buffer (Stratagene). 1 unit of *Pfu* polymerase (Stratagene) was added, and then the reaction was covered with mineral oil and placed in a Perkin-Elmer Thermocycler. Conditions were programmed to heat the solution to 95 °C for 5 min and then to cycle it to 60 °C for 1 min, 74 °C for 1.5 min, and then 95 °C for 1 min (for a total of 35 cycles), followed by a final incubation at 74 °C for 10 min. The reactions were chloroform-extracted, and the amplified DNA was ethanol-precipitated. After washing in 70% ethanol and drying, the DNA was cleaved with the appropriate restriction enzymes using the manufacturer’s recommended conditions. The DNA was then fractionated by gel electrophoresis on 2% low melt agarose gels, and the appropriate size band was excised and purified. The three restricted PCR products isolated consisted of a 0.83-kb *SalI*/*NspI* fragment, a 0.28-kb *BamHI*/*Vgl* fragment, a 0.28-kb *NspI*/*SalI* fragment, and a 0.14-kb *NheI/*BamHI* fragment. These fragments were ligated together with equimolar amounts of a 1.05-kb *SalI*/*NspI* fragment, a 1.3-kb *NdeI*/*NheI* fragment, and the 3-kb vector pBlueScript SK+ (Stratagene) cleaved with *SalI*/*BamHI*. Restriction fragment analysis was used to identify a full-length nit-6 cDNA. Dideoxy sequencing was carried out over the PCR-derived portions and ligation boundaries to ensure no mutations had occurred. The resulting clone was designated pNiR-3, and the insert was named nit6.5. The full-length nit-6 cDNA (nit6.5) was subcloned as a single fragment from pNiR-3 into expression vector pET11a via *NdeI*/*BamHI* cleavage, yielding plasmid pETnit6.5. In this construct, the ATG translational start codon of nitrite reductase falls within the *NdeI* site, and the stop codon lies upstream from the *BamHI* site.

**PCR Subcloning**—Standard reaction conditions were used for PCR amplification of cloned DNA (22). These techniques were routinely used to alter flanking sequences and introduce novel restriction sites to facilitate subcloning or otherwise manipulate DNA. DNA containing

![Diagram](http://www.jbc.org/Downloaded from)
the E. coli cysG gene was amplified by PCR from plasmid pBSM10 kindly provided by Nicholas Kredich of Duke University Medical Center (23), using two oligomeric primers flanking the gene. One primer was designed to hybridize upstream of the Shine-Dalgarno sequence of the cysG gene. This primer introduced a BamHI site. The other primer hybridized just downstream of the 3′ end of the gene. This primer introduced a SalI site and BamHI site. The resulting 1.8-kb product was cleaved with BamHI and isolated from a 1% low melt agarose gel following gel electrophoresis. This fragment was ligated into the BamHI site downstream of the nit6.5 insert in pETnit6.5 (Fig. 2). Restriction analysis was used to identify clones containing the cysG gene in the same orientation as the nit-6 cDNA. The resulting plasmid was designated pETnit6.5cysG, and the insert was named nit6.5cysG. The nit6.5cysG insert was also subcloned into vector pTRC99A for expression in E. coli strain JC3878, a nirB− (deletion) strain lacking the gene encoding the E. coli NADH-nitrite reductase. Strain JC3878 was kindly provided by Jeffrey Cole of the University of Birmingham (United Kingdom). Cloning into vector pTRC99A required a new restriction site at the 5′ end of nit6.5cysG in order to ligate the insert in the proper orientation and reading frame for protein expression. Using a primer designed to introduce an NcoI site that hybridized to the 5′ end of the nit-6 cDNA and a primer designed to hybridize downstream of the NspV site in the nit-6 cDNA, PCR was performed to amplify the intervening DNA between these sites within nit6.5cysG, creating a 84-kb fragment with an NcoI site at the 5′ end. After chloroform extraction of the reaction mixture, the DNA was ethanol-precipitated, dried, and dissolved in TE. The DNA was then digested with NcoI, followed by digestion with NspV. The digested DNA was fractionated by gel electrophoresis on a 1.5% low melt agarose gel, followed by excision and purification. The NcoINspV fragment was ligated together with an NspV/SalI fragment of pETnit6.5cysG (containing a 2.76-kb portion of the nit-6 cDNA and the entire cysG gene) into expression vector pTRC99A cleaved with NcoI and SalI. The resulting plasmid was designated pTRCnit6.5cysG.

Partial constructs of the nit-6 cDNA were made in conjunction with restriction digest products of a single PCR reaction. Two oligonucleotide primers were synthesized. One primer hybridized 0.38 kb downstream of the EclXI site within nit6.5. This primer was designed to introduce an early stop codon and BamHI site within the cDNA. Another primer was designed to introduce an Ndel site and translational start site about 80 nucleotides upstream of the EclXI site. The intervening sequence between the primers was amplified using PCR. The reaction mixture was chloroform-extracted, and the DNA was ethanol-precipitated and dissolved in TE. Half of the product was digested with EclXI and BamHI. The other half was digested with EclXI and Ndel. The EclXI/BamHI-digested DNA was ligated to a 4.12-kb fragment of pETnit6.5 cleaved with EclXI and BamHI (this effectively removed more than half of the nit-6 cDNA at its 3′ end). The resulting plasmid was designated pETSN, for 5′ (N-terminal) construct. The Ndel/EclXI-digested PCR product was ligated to the 5.48-kb fragment of pETnit6.5 digested with NdeI and EclXI (thereby removing about 1.12 kb of DNA at the 5′ end of the nit-6 cDNA), producing the plasmid designated pETS3C, for 3′ (C-terminal) construct. Fig. 2 displays a restriction map of the cDNA constructs. The sites shown are restriction sites exploited in DNA manipulations and subcloning.

Site-directed Mutagenesis—Site-directed mutagenesis was achieved using the Altered Sites kit from Promega. The full-length nit-6 cDNA (nit6.5) was ligated into the vector pALTER-1 (supplied with the kit) via the BamHI and SalI site introduced at the ends of the cDNA during its construction (Fig. 1). Mutagenic DNA oligomeric primers were synthesized and purified, as described previously (21). The design of these oligonucleotides is shown in Fig. 5. A 0.1-pmol aliquot of oligonucleotide was phosphorylated using T4 polynucleotide kinase, as recommended by the manufacturer (Boehringer Mannheim). Mutagenesis was carried out as described in the Altered Sites manual. Mutagenized plasmid DNA was analyzed by direct nucleotide sequence determination or, where applicable, by restriction endonuclease analysis (see Fig. 5), followed by direct nucleotide sequence determination of the DNA in the region of the mutation. The DNA mutated within the region encoding the FAD/NAD-binding domain of nitrite reductase was transferred to plasmid pTRCnit6.5cysG as a 0.3-kb fragment. This fragment was isolated by cleaving the DNA with restriction endonuclease NspV and EclXI, followed by electrophoresis and excision from a 3% low melt agarose gel. This fragment was ligated into the 9.57-kb NspV/EclXI-digested fragment of DNA from plasmid pTRCnit6.5cysG (which lacked the comparable segment of DNA). The DNA mutated within the region encoding the (Fe₄S₄)₅-siroheme-binding domain of nitrite reductase was subcloned into plasmid pTRCnit6.5cysG as a 0.3-kb fragment obtained by cleaving with restriction endonucleases AatII and SphI, followed by electrophoresis and excision from a 3% low melt agarose gel. The fragment was ligated together with a 6.3-kb Sall/AatII fragment and a 4-kb SalI/AatII fragment from pTRCnit6.5cysG (a single fragment derived from an AatII/SphI digest was not applicable because there are several AatII sites within the nit-6 cDNA).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—7% SDS-polyacrylamide gels were prepared and gel electrophoresis was performed as described previously (24). Prestained protein molecular weight markers were purchased from Boehringer Mannheim.
Functional Analysis of Nitrite Reductase from N. crassa

EXPERIMENTAL PROCEDURES

The nitrite reductase gene of N. crassa (nit-6) was isolated and sequenced (5, 24). To address the possibility that some fraction of the nit-6 polypeptide expressed in E. coli was incorrectly folding or not properly associating into its correct oligomeric form, plasmid pTRCnit6.5cysG was transformed into E. coli strain CB926 (25). Strain JCB3878 lacks the lac F' gene necessary to repress expression from pET11a, but pTRC99A carries the lac F' gene on the plasmid itself. pTRCnit6.5cysG was transformed into E. coli strain JCB3878, and the cells were induced for protein expression. Extracts of such cells possessed elevated levels of NADPH-nitrite reductase activity almost 3 times higher than from cells expressing the construct without cySG (Table III).

Under anaerobic conditions, the host E. coli nirB gene product, NADH-nitrite reductase, becomes expressed. This enzyme also requires siroheme, and therefore its expression may reduce cofactor availability to the N. crassa nitrite reductase. To circumvent this, the nit6.5cysG insert was subcloned into expression vector pTRC99A, resulting in plasmid pTRCnit6.5cysG and grown under anaerobic conditions. Extracts of such cells possessed elevated levels of NADPH-nitrite reductase activity almost 3 times higher than transformed cells otherwise identical.

Results

Nitrite reductase is a dimeric enzyme that requires siroheme as a prosthetic group and FAD and NADPH as redox cofactors. The FAD/NADPH binding domain of the enzyme is likely to be the rate-limiting step in siroheme protein production (23). Cell-free extracts from cultures of E. coli strain CB926 transformed with pETnit6.5cysG and grown under anaerobic conditions demonstrated higher levels of NADPH-nitrite reductase activity than those of the vector control. This suggests that one or more proteins involved in siroheme biosynthesis are induced under these conditions.

The nit-6 gene product (Fig. 3). These residues compose the putative FAD/NAD-binding domain of the protein but lack the putative (Fe4S4)2-siroheme-binding domain. E. coli strain CB926 growing at 37°C, and the cells were induced for protein expression under anaerobic or aerobic conditions. Cell-free extracts from these cultures did not possess detectable native or partial enzyme activity, although successful expression of protein was deduced by immunoblot analysis.

However, enzyme activity was detected in cell-free extracts from transformed cells grown at room temperature (Table II). Higher levels of nitrite reductase activity were obtained in extracts of cells grown anaerobically in minimal media; therefore, these conditions were used routinely when expressing protein in E. coli. Activity within extracts was further increased by introduction of the cySG gene into the nit-6 expression plasmid and introduction of plasmid pGroESL (25) into E. coli host JCB3878 (Table III). The cySG gene of E. coli and S. typhimurium encodes uroporphyrinogen III methyltransferase, which converts uroporphyrinogen III into siroheme. This enzyme is likely to be the rate-limiting step in siroheme production (23).

Equivalent amounts of protein from cell-free extracts were fractionated on each gel (20 μg/lane). Fractionated protein was transferred to a nitrocellulose filter using an LKB Electro-Blot apparatus as described previously (24). Filters were air-dried and stored at 4°C. Immunoblot analysis was performed using an immunoblot assay kit (goat anti-rabbit IgG (H + L) alkaline phosphatase) from Bio-Rad. Kit components were used as recommended by the manufacturer. The filters were probed with primary antibody specific for N. crassa nitrite reductase. This antibody was generated and purified as described previously (10, 19, 24). Antibody concentrations of 1% were used immediately or stored frozen at −20°C.

The filters were probed with primary antibody specific for N. crassa nitrite reductase. This antibody was generated and purified as described previously (10, 19, 24). Antibody concentrations of 1% were used immediately or stored frozen at −20°C.
Nitrite reductase activity in extracts from E. coli cultures expressing the nit-6 gene. Shown are different enzyme activities within cell-free extracts from E. coli cultures expressing the full-length nit-6 cDNA either alone or with the cysG gene introduced into the expression construct, when nirB deletion strain JCB3878 was used as host for expression, and pGroESL was introduced into the host. Cyt/Nit is the ratio of NADPH-cytochrome c reductase activity to NADPH-nitrite reductase activity. (All E. coli cultures were grown under anaerobic conditions at room temperature.)

| Plasmid(s) | E. coli host strain | NADPH-nitrite reductase activitya | Dithionite-nitrite reductase activitya | NADPH-cytochrome c reductase activityb | Cyt/Nit  |
|------------|---------------------|----------------------------------|--------------------------------------|----------------------------------------|---------|
| pETnit6.5  | CB926               | 99.7                             | 4.1                                  | 1190                                   | 11.9    |
| pET5N      | JCB3878 (nirB−)     | 282                              | 6.2                                  | 1396                                   | 4.95    |
| pET3C      | JCB3878 (nirB−)     | 836                              | 29.3                                 | 7137                                   | 8.5     |

* Expressed as nmol of nitrite reduced per min per mg of protein.
  b Expressed as nmol of cytochrome c reduced per min per mg of protein.

Increasing nitrite reductase activity in E. coli extracts

The other two mutations were located within the region of DNA encoding the (Fe₄S₄)-siroheme-binding domain (Fig. 5). In one of these mutations, Ser-755 was replaced with alanine, generating plasmid pTRC.C757A. A second mutation in this region replaced Cys-757 with an alanine, generating plasmid pTRC.C757A. Each of these mutations was constructed to determine whether one or both of these residues was essential to the function of this domain. The third mutation involved two amino acid changes; Ser-755 was replaced with Cys and Cys-757 was replaced with Ser, generating plasmid pTRC.C757C/C757S, in order to determine if the position of these two residues relative to one another was essential for function.

Each of the mutant plasmid constructs was transformed along with pGroESL and expressed in host strain JCB3878. Cell-free extracts were prepared and assayed for NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase activities (Table IV). Based on these enzyme assays, none of the altered amino acid residues appeared to be essential for NADPH-cytochrome c reductase activity (Table IV). Only residue Cys-757 was essential for NADPH-nitrite reductase and dithionite-nitrite reductase activity. The position of residue Cys-757 and Ser-755 relative to each other was also essential for NADPH-nitrite reductase and dithionite-nitrite reductase activity. Cys-331 and Ser-755 were not essential for NADPH-nitrite reductase, NADPH-cytochrome c reductase, or dithionite-nitrite reductase activity (Table IV). The S757A mutation did abolish NADPH-nitrite reductase and dithionite-nitrite reductase activities in extracts from cultures grown under anaerobic conditions (data not shown). A protein band cross-reactive with the anti-nitrite reductase antibody with an Mr of approximately 136,000 was detected in each of the mutant extracts as shown by immunoblot analysis (Fig. 4).
The association of NADPH-cytochrome c reductase activity with the FAD-/NAD-binding domain independently of the (Fe₄S₄)‑siroheme-binding domain and the association of the dithionite-nitrite reductase activity with the region of the protein possessing the (Fe₄S₄)‑siroheme-binding domain independently of the FAD-/NAD-binding domain clearly defines these domains as the functional entities responsible for the partial activities of N. crassa nitrite reductase.

The ratio of NADPH-cytochrome c reductase activity to NADPH-nitrite reductase activity (Cyt/Nit, Table IV) indicates the relative effect that an amino acid replacement has on the functioning of the FAD-/NAD-binding domain in comparison with the (Fe₄S₄)‑siroheme-binding domain. The Cyt/Nit ratio of native nitrite reductase isolated from N. crassa increases during final purification of the protein. This increase is attributed to the instability of the nitrite-reducing function of the enzyme (associated with the (Fe₄S₄)‑siroheme cluster) during the purification procedure (7). Conditions resulting in the highest heterologous expression of NADPH-nitrite reductase enzyme activity within cell-free extracts of E. coli (anaerobic growth, cysG downstream of the nit-6 cDNA in pTRC99A, and pGroESL co-transformed into the host strain JCB3878) yields a Cyt/Nit ratio virtually identical to that found in cell-free extracts derived from nitrate-induced N. crassa mycelia (8.5 versus 8.6, respectively (Table III). In this aspect, the E. coli expression system under optimal conditions yields N. crassa nitrite reductase with the functional properties of N. crassa nitrite reductase obtained from its native source.

Several amino acid residues within nitrite reductases from fungi and E. coli have been suggested to play vital roles in nitrite reductase activity, based on inhibitor studies and/or sequence conservation and location within putative functional domains (9, 10). These residues were chosen for amino acid replacements within N. crassa nitrite reductase in an effort to establish their functional importance by direct biochemical evidence. Replacement of the highly conserved residue Cys-331 did not abolish NADPH-nitrite reductase activity, NADPH-cytochrome c reductase activity, or dithionite-nitrite reductase activity. However, extracts of cultures expressing pTRC.C331A showed a reduction in the Cyt/Nit ratio compared with that of the wild-type full-length construct expressed under the same conditions (5.7 versus 8.5 (Table IV)).

The dithionite-nitrite reductase activity within extracts expressing pTRC.C331A was diminished to a lesser extent than either NADPH-dependent activity. Apparently, this mutation influences electron flow within the FAD-/NAD-binding domain more than within the (Fe₄S₄)‑siroheme domain. Whatever its function, the highly conserved Cys-331 is not essential for the electron transfer during catalysis.

Expression of the S755A mutant protein yielded extracts possessing NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase activity, demonstrating that Ser-755 is not essential for the electron transfer reactions of nitrite reductase. The Cyt/Nit ratio for S755A increased from 5.5 to 26, indicating a greater impairment of function in the nitrite-reducing properties of the enzyme compared with its cytochrome c-reducing properties. Perhaps Ser-755 plays a peripheral role in stability of the nearby (Fe₄S₄)‑siroheme cluster.

In the C757A mutant, Cys-757, one of the four Cys residues that coordinates the Fe₄S₄ cluster, is replaced with Ala. Extracts from cultures expressing pTRC.C757A were essentially devoid of NADPH-nitrite reductase or dithionite-nitrite reductase activity (Table IV). Cys-757 is thus essential for the nitrite-reducing function of nitrite reductase but is not essential for NADPH-cytochrome c reductase activity (Table IV). Recent crystallographic data show that the bridging ligand in the (Fe₄S₄)‑siroheme cluster of E. coli sulfite reductase is Cys-483 (13), which corresponds to Cys-761 in the N. crassa nitrite reductase, not Cys-757.

The relative position of Ser-755 and Cys-757 is essential for nitrite reductase activity, since extracts from cultures expressing pTRC.S755C/C757S lack detectable NADPH-nitrite reductase and dithionite-nitrite reductase activity (Table IV). NADPH-cytochrome c reductase activity in the S755C/C757S double mutant is essentially unaffected.

Several factors affect the activity of N. crassa nitrite reductase during expression in E. coli. Coomassie Blue staining of proteins in extracts fractionated by SDS-polyacrylamide gel
electrophoresis demonstrated that the different levels of enzyme activity under various growth conditions are not simply due to the level of protein being expressed (data not shown). The lowered temperatures necessary to obtain NADPH-nitrite reductase, NADPH-cytochrome c reductase, and dithionite-nitrite reductase expression under all conditions examined suggest that the enzymatic activities of the nil-6 encoded protein are unstable at 37°C. Perhaps this thermal instability of N. crassa NAD(P)H-nitrite reductase.

**TABLE IV**

| Plasmid       | NADPH-Nir<sup>a</sup> | Dithionite-Nir<sup>a</sup> | NADPH-Cyt. c<sup>b</sup> | CRM<sup>c</sup> | Cyt/Nir<sup>d</sup> |
|---------------|-----------------------|---------------------------|-------------------------|-----------------|----------------------|
| pTRC.C331A    | 357                   | 14.6                      | 2038                     | 80              | 5.7                  |
| pTRC.S755A    | 91                    | 2.1                       | 2363                     | 55              | 26.0                 |
| pTRC.C757A    | 0                     | 0                         | 1076                     | 30              |                      |
| pTRC.S755C/C757S | 0                  | 0                         | 1445                     | 52              |                      |

<sup>a</sup> NADPH- or dithionite-nitrite reductase specific activity (nmol of nitrite reduced per min per mg of protein).

<sup>b</sup> NADPH-cytochrome c reductase specific activity (nmol of cytochrome c reduced per min per mg of protein).

<sup>c</sup> Cross-reacting material (based on densitometry readings of immunoblots probed with anti-nitrite reductase antibody).

<sup>d</sup> Ratio of NADPH-cytochrome c reductase to NADPH-nitrite reductase activity.
crassa nitrite reductase is related to protein folding or assembly. Since all three activities are affected by temperature, temperature sensitivity is not localized within a single domain. It should be noted that N. crassa grows well at 25°C but poorly above this temperature.

The increase in nitrite reductase activities resulting from nit-6 expression in E. coli under anaerobic conditions versus aerobic conditions may result from a sensitivity of siroheme to aerobic conditions. Aerobic conditions are more detrimental to aerobic conditions may result from a sensitivity of siroheme to aerobic conditions. Aerobic conditions limited siroheme acquisition by the heterologously expressed NADPH-nitrite reductase activity (data not shown). However, expression of pTRCnit6.5cysG in strain E. coli under anaerobic conditions yielded extracts with NADPH-nitrite reductase activity (data not shown). Under aerobic conditions limited siroheme acquisition by the heterologously expressed NADPH-nitrite reductase activity (Table II and III). Under aerobic conditions the effect of introducing cysG into plasmid pETnit6.5 was much greater (a 3-fold increase in nitrite reductase activity was seen in extracts from aerobically grown cells under optimum conditions of cysG (data not shown)). However, expression of pTRCnit6.5cysG in strain JCB3878 under anaerobic conditions yielded extracts with NADPH-nitrite reductase activity almost 3-fold higher than that achieved by expressing pETnit6.5cysG in strain CB926 under the same conditions. This was predominantly due to a specific increase in the nitrite-reducing function of the protein expressed, since the specific activity for NADPH-cytochrome c reductase was only increased by 15% (Table III). A possible reason is that competition for siroheme manifested by the presence of E. coli NADH-nitrite reductase under anaerobic conditions limited siroheme acquisition by the heterologously expressed N. crassa enzyme.

The transformation of pGroESL into cells in which pTRCNit6.5cysG was expressed resulted in extracts with increased NADPH-nitrite reductase, dithionite-nitrite reductase, and NADPH-cytochrome c reductase activities (Table III). This elevation in activity and the similarity of the Cyt/Nit ratio (Table IV) to that of the enzyme isolated from N. crassa indicates that GroES and/or GroEL may assist in the proper folding and/or assembly of N. crassa nitrite reductase within E. coli.

This study provides direct evidence that N. crassa NAD(P)H-nitrite reductase possesses discrete functional domains and that these domains are laid out in a linear fashion along the polypeptide. The N-terminal portion of the polypeptide is responsible for the NAD(P)H- and FAD-dependent functions of the enzyme, while the C-terminal portion contributes the nitrite-reducing capabilities. Thus, the sequence of electron transfer in N. crassa nitrite reductase from NAD(P)H to nitrite is reflected in the domain organization of the polypeptide subunit from N-terminal to C-terminal and in the nucleotide sequence of the nit-6 gene from its 5'-end to its 3'-end. Nevertheless, it would be misleading to assume that the nitrite reductase holoenzyme in its catalytically active homodimeric state mediates electron transfer down two parallel (or antiparallel) tracks defined by pairs of subunits. It is equally plausible that electron transfer takes place from the FAD/NAD-binding domain of one subunit to the juxtaposed (Fe4S4)-siroheme-binding domain of the other subunit.

In conclusion, the highly conserved Cys-331 and the conserved Ser-755 are not essential for native nitrite reductase activity or partial activities, whereas Cys-757 is essential for the nitrite-reducing catalytic functions of the enzyme.

Acknowledgments—We thank Nicholas Kredich for supplying plasmid pRSM10, Jeffrey Cole for supplying strain JCB3878, David Tolbert for assistance with densitometry, and Jeff Skinner for assistance with site-directed mutagenesis.

REFERENCES

1. Crawford, N. M. (1995) Plant Cell 7, 859–868
2. Crawford, N. M., and Campbell, W. H. (1990) Plant Cell 2, 829–835
3. Dunn-Coleman, N. S., Smarella, J., Jr., and Garrett, R. H. (1984) Int. Rev. Cytol. 92, 1–49
4. Garrett, R. H., and Amy, N. K. (1978) Adv. Microb. Physiol. 18, 1–65
5. Lafferty, M. A., and Garrett, R. H. (1974) J. Biol. Chem. 249, 7555–7567
6. Prochazka, K. N., and Garrett, R. H. (1981) J. Biol. Chem. 256, 9711–9717
7. Vega, J. M. (1976) Arch. Microbiol. 109, 237–242
8. Vega, J. M., and Garrett, R. H. (1975) Proceedings of the Sixth Congress of the Spanish Society of Biochemistry, Seville, Spain, p. 108
9. Campbell, W. H., and Kinghorn, J. R. (1990) Trends Biochem. Sci. 15, 315–319
10. Exley, G. E., Clandine, J. D., and Garrett, R. H. (1993) J. Bacteriol. 175, 2379–2392
11. Johnstone, I. L., McCabe, P. C., Grevet, P., Gurr, S. J., Cole, G. E., Brow, M. A. D., Unkles, S. E., Clutterbuck, A. J., Kinghorn, J. R., and Innis, M. A. (1990) Gene (Amst.) 90, 181–192
12. Peakman, T., Crouzet, J., Mayaux, J. P., Busby, S., Mohan, S., Harborne, N., Wootton, J., Nicolson, R., and Cole, J. (1990) Eur. J. Biochem. 191, 315–323
13. Crane, B. R., Siegel, L. M., and Getzoff, E. D. (1995) Science 270, 59–67
14. Kinghorn, J. R., and Campbell, E. (1989) in Molecular and Genetic Aspects of Nitrate Assimilation (Wray, J. L., and Kinghorn, J. R., eds) Oxford University Press, New York, pp. 385–403
15. Siegel, L. M., and Wilkerson, J. O. (1989) in Molecular and Genetic Aspects of Nitrate Assimilation (Wray, J. L., and Kinghorn, J. R., eds) Oxford University Press, New York, pp. 263–283
16. Back, E., Burkhart, W., Moyer, M., Privalle, L., and Rothstein, S. (1988) Mol. Gen. Genet. 212, 20–36
17. Lahners, K., Kramer, V., Back, E., Privalle, L., and Rothstein, S. (1991) Plant Physiol. 88, 741–746
18. Kornberg, R., Hirt, R., and Hachtel, W. (1992) Mol. Gen. Genet. 231, 411–416
19. Ostrowski, J., Wu, J.-Y., Ruesger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. (1989) J. Biol. Chem. 264, 15726–15737
20. Davis, R. H., and De Serres, F. J. (1970) Methods Enzymol. 17A, 79–143
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Jakobsen, T., Gussow, D., and Jones, P. T. (1991) in PCR: A Practical Approach (Quirke, P., and Taylor, G. R., eds) Oxford University Press, New York, 187–214
23. Wu, J.-Y., Siegel, L. M., and Kredich, N. M. (1991) J. Bacteriol. 173, 325–333
24. Hurlbut, B. K., and Garrett, R. H. (1988) Mol. Gen. Genet. 211, 35–40
25. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44–47
Functional Dissection and Site-directed Mutagenesis of the Structural Gene for NAD(P)H-Nitrite Reductase in *Neurospora crassa*

James D. Colandene and Reginald H. Garrett

*J. Biol. Chem.* 1996, 271:24096-24104.
doi: 10.1074/jbc.271.39.24096

Access the most updated version of this article at http://www.jbc.org/content/271/39/24096

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 20 references, 8 of which can be accessed free at http://www.jbc.org/content/271/39/24096.full.html#ref-list-1