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Nitrate reductase of *Neurospora crassa*: Characterization of its gene, *nit-3*, and structural studies of its heme-binding domain

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The Ohio State University, 1992
NITRATE REDUCTASE OF Neurospora crassa:
CHARACTERIZATION OF ITS GENE, nit-3, AND STRUCTURAL STUDIES
OF ITS HEME-BINDING DOMAIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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* * * * *

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To my family
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CHAPTER I
LITERATURE REVIEW

The utilization of inorganic nitrogen (N$_2$ or NO$_3$) from the environment by living organisms requires its conversion into a more metabolically suitable organic form and is accomplished through either nitrogen fixation or nitrate assimilation. Whereas nitrogen fixation (Merrick 1992) occurs in some procaryotes, like Klebsiella, and cyanobacteria and in certain plants (e.g. legumes) which have evolved a symbiotic relationship with nitrogen-fixing bacteria, many fungi, algae, bacteria and economically important plants such as barley, wheat and maize obtain their nitrogen via nitrate assimilation. These nitrate assimilating organisms, in turn, supply organic nitrogen to other living things (e.g. humans) that are unable to metabolize inorganic nitrogen. In a global sense, the significance of nitrate assimilation far exceeds that of nitrogen fixation in the production of organic nitrogen, and a large proportion of the energy derived from photosynthesis is spent driving the nitrate assimilatory process (Guerrero et al. 1981). Consequently, because of its essential biological and ecological roles, a great deal of research has been invested in studying the nitrate assimilatory pathway.

In terms of economic importance, an understanding of the nitrate assimilatory process in plants is particularly of value to man. Unfortunately, however, the use of
plants as experimental systems for genetic analysis and manipulation is often difficult and ill-defined. Lower eucaryotes such as the filamentous fungi and yeasts, on the other hand, frequently have well-characterized genetics (Fincham et al. 1979) and established biological techniques, thus providing a system that is more amenable to experimentation. One such lower eucaryote is the filamentous fungus *Neurospora crassa* which has been used considerably in the genetic and biochemical analyses of a number of metabolic pathways including nitrate assimilation.

In its vegetative form, the filamentous ascomycete *Neurospora crassa* exists as a mycelial mass which is haploid and composed of hyphae, the basic structural units of the fungus. Hyphae are branched, cylindrical cells that are usually multinucleated as a result of incomplete cross walls between the cells. Vegetative growth occurs rapidly at the hyphal tips; aerial hyphae give rise to conidiophores which produce two kinds of asexual spores called macroconidia and microconidia. Macroconidia are abundant, often have 3 to 5 nuclei per conidium, and possess a distinctive orange color, hence the reason for the organism's common name, the pink bread mold. Conversely, microconidia are smaller in size, have lower viability and usually are uninucleated, a property that makes them useful in certain genetic experiments. Provided that no differences in incompatibility factors are present, hyphal fusions may occur between genetically different mycelia giving rise to mycelia with two or more different kinds of nuclei. Those strains that contain genetically distinct haploid nuclei are termed heterokaryons.

Upon conditions that impede vegetative growth such as nitrogen starvation, *Neurospora* will enter into the sexual phase of its life cycle by producing nascent fruiting
bodies, or protoperethecia (the female structures). As a heterothallic hermaphrodite, the sexual stage of Neurospora involves strains of two mating types \( A \) and \( a \). Either mating type is able to produce both male gametes (conidia) and female gametes. Fertilization occurs when a long specialized hypha called the trichogyne projects from the protoperethecium and comes in contact with a conidium of the opposite mating type. The conidial nucleus is transported down into the protoperethecium, and the parental nuclei separately undergo several rounds of mitotic division prior to nuclear fusion. Once fusion has taken place, the short-lived diploid cell containing the nuclei of each mating type immediately enters into meiosis and then one mitotic division to produce eight haploid cells. These haploid cells are termed ascospores and enclosed within an ascus sac. A perethecium, or mature fruiting body, contains several hundred asci. The ascospores may stay dormant for a long period of time and can withstand harsh circumstances, germinating into mycelia only after a heat shock or similar stress.

Although Neurospora is a heterotroph, it has few nutritional requirements and may easily be cultured in the laboratory on a well-defined nutrient medium that consists of a carbon source, inorganic nitrogen, biotin and various mineral salts. More importantly, auxotrophic mutations can be readily isolated and identified by altering the composition of the standard minimal medium (i.e., medium having the basic nutrients required to sustain wild-type Neurospora growth) as was first demonstrated by Beadle and Tatum (1941). Since their pioneering work on metabolic pathways in Neurospora crassa that eventually lead to the "one-gene, one-enzyme" hypothesis, a great number of biochemical mutants have been isolated in Neurospora, including those involved in
nitrate assimilation. Furthermore, due to their nutritional versatility, the use of auxotrophs for metabolic studies has not only been very valuable in elucidating the sequence of enzymatic reactions in a metabolic pathway, but has also contributed significantly to the understanding of gene-enzyme relationships, promoters, protein-protein and DNA-protein interactions, enzyme structure and activity, and regulatory mechanisms in eucaryotes.

Responses to environmental changes such as nutritional availabilities and deficiencies require a highly controlled mode of gene expression. A wealth of information on genetic regulation has been obtained with fungi by analyzing the metabolism of a specific compound which must be used by the organism as the sole source of an essential element, *e.g.*, phosphorus, carbon, sulfur or nitrogen. That is, depending upon the nutritional source available for a particular essential element, the organism will synthesize certain enzymes, or more often, a whole set of enzymes in order to metabolize the compound. Expression of these sets of enzymes requires both regulatory and structural genes that collectively constitute elaborate metabolic circuits which, depending upon the nutritional source present, may regulate enzyme formation in a constitutive, repressive or inducible manner.

Genetic regulation has been studied extensively in *Neurospora*. Among the metabolic circuits that have been examined, the phosphorus, quinic acid, sulfur and nitrogen circuits are four of the best characterized in terms of biochemistry and genetics, and are of particular interest. The regulation of gene expression in all four circuits appears to occur at the transcriptional level, although there exist slight variations in the
overall hierarchy among the regulatory genes of a particular circuit. In addition, two or more metabolic circuits may independently regulate the expression of a single structural gene. For example, an extracellular alkaline protease gene has been shown to be controlled by three distinct regulatory genes from the sulfur, carbon and nitrogen circuits (Hanson and Marzluf 1975).

The phosphorus acquisition circuit comprises a cascade or relay of regulatory genes that are ordinarily repressed and only come into play when Neurospora is starved for phosphate. The regulatory cascade, deduced mainly from genetic analyses of epistatic and dominant interactions of mutant alleles, consists of four genes called nuc-2+, preg+, pgov+ and nuc-1+ that are involved in the synthesis of a number of enzymes important in the uptake of phosphate. While nuc-1+ acts in a positive manner by directing the expression of various unlinked structural genes, the nuc-2+, preg+, and pgov+ genes are negative regulators. According to the current model (Littlewood et al. 1975; Metzenberg 1979), the absence of phosphate or its derivative allows for the activation of nuc-2+ which then inhibits the activities of preg+ and/or pgov+. Normally, the preg+ and pgov+ genes together act to suppress the action of nuc-1+. Upon phosphate starvation, they no longer antagonize nuc-1+, and repression of the unlinked structural genes is lifted, allowing the production of phosphorus acquisition enzymes. Molecular characterization of the nuc-1+ gene (Kang and Metzenberg 1990) has revealed that the predicted NUC-1 protein sequence is homologous to two regulatory proteins, PHO2 and PHO4, which jointly have the analogous function to NUC-1 in phosphorus acquisition of Saccharomyces cerevisiae. Postulated to be a DNA-binding transcriptional
activator, NUC-1 may possess an amphipathic helix-turn-helix domain and three possible transcriptional activating motifs in the forms of glutamine-rich, proline-rich, and acidic regions.

Although there is a lack of conclusive evidence for the mechanism by which these regulatory genes interact with each other, the constitutive expression of *nuc-1 +* mRNA and complementation of a *nuc-2* mutation by overexpression of *nuc-1 +* (Kang and Metzenberg 1990) support the notion that the NUC-1 protein is produced at a constant level, and that the regulatory mechanism entails the titration of a regulatory protein by the protein preceding it in the cascade (Metzenberg and Chia 1979), perhaps through direct protein-protein interactions. Moreover, based on studies with *pgov +* constitutive mutants, the role of *pgov +* may also involve sensing the presence of phosphate or its derivatives in the circuit (Stallwag and Metzenberg 1984).

As the sole carbon source for *Neurospora*, quinic acid is a poor alternative to sucrose or glucose. Yet, the quinic acid catabolic system is one of the best studied of the metabolic pathways because it is a rare eucaryotic example of a control mechanism that is operon-like in genetic organization and regulation. The quinic acid catabolic pathway consists of seven tightly linked genes, five of which are the structural genes, *qa-2, qa-3, qa-4, qa-x* and *qa-y*, and two are the regulatory genes, *qa-1F* and *qa-1S*, that collectively form the *qa* gene cluster (Giles et al. 1985). The structural genes are expressed as discrete mRNA transcripts at very low basal levels under repressed conditions and become highly induced in the presence of quinic acid (Patel et al. 1981). Located at one end of the cluster, the divergently transcribed *qa-1F* and *qa-1S* regulatory
genes are capable of working in trans as well as cis and encode, respectively, an activator and a repressor protein which are also constitutively present at low levels. The prevailing model for gene regulation in the qa gene cluster (Geevers et al. 1989) postulates that the qa-1S repressor protein, indirectly or directly, down regulates the expression of the qa-1F gene, resulting in the observed basal level expression of the structural genes. Upon specific induction by quinic acid, the derepression of qa-1F stimulates the syntheses not only of the qa structural genes but also of itself (Patel and Giles 1985) and of qa-1S. The qa-1F activator has been shown to bind via a putative zinc finger domain to several conserved short promoter sequences containing partial dyad symmetry located upstream of each qa gene of the cluster. One particular site is located within the region common to both regulatory genes (Baum et al. 1987), further substantiating prior genetic evidence that qa-1F regulates its own expression and that of qa-1S. The mechanism by which qa-1S repressor acts is still uncertain (Giles et al. 1985).

In comparison to the quinic acid catabolic circuit, the sulfur circuit appears to be less complex in genetic organization. Interestingly, unlike the phosphorus, quinic acid and nitrogen circuits which all produce freely diffusible internuclear regulatory proteins, the sulfur circuit illustrates an unusual case of intranuclear control by a regulatory gene product (i.e., action of the regulatory gene product appears to be confined to the nucleus in which it is synthesized; Metzenberg and Ahlgren 1970). In the sulfur metabolic circuit, an assortment of sulfur catabolizing enzymes that include two distinct sulfate permeases (Marzluf 1970a,b), arylsulfatase (Metzenberg and Ahlgren 1970; Paietta 1991)
and a methionine permease (Pall 1971) are synthesized when sulfur is limited. The expression of the unlinked structural genes which encode these enzymes is controlled by three unlinked regulatory genes, cys-3, scon-1, and scon-2, that together make up the sulfur catabolic circuit. Genetic studies have suggested that the cys-3 regulatory gene turns on the expression of the various structural genes (Marzluf and Metzenberg 1968) as well as autoregulates its own expression (Paietta et al. 1987). More recent characterization of cys-3 (Paietta et al. 1987) has revealed that the CYS-3 protein is a transcriptional activator with a sequence-specific DNA-binding domain which is comprised of neighboring basic and leucine zipper regions (Fu et al. 1989b; Fu and Marzluf 1990c). The leucine zipper is presumed to orient the basic region and facilitate CYS-3 binding to the promoter regions of the unlinked sulfur structural genes (Kanaa’n and Marzluf 1991) such as the sulfate permease II gene, cys-14 (Ketter and Marzluf 1988; Ketter et al. 1990), and of cys-3 itself (Fu and Marzluf 1990c), thereby supporting the claim that the CYS-3 protein is autoregulatory.

It has been genetically inferred that the other two regulatory genes, scon-1 and scon-2, exert negative effects on the circuit (Burton and Metzenberg 1972) by regulating the expression of cys-3 (Fu et al. 1989b; Paietta, 1990). Although scon-1 displays nucleus-limited function as shown by constitutively derepressed sulfur enzyme expression (Burton and Metzenberg 1972), scon-2 does not seem to have this effect. Recent molecular analysis of the scon-2 gene has shown that scon-1 negatively controls the expression of scon-2 (Paietta 1990). Therefore, the basic model for the genetic regulation of the sulfur circuit proposes that the scon-1 gene monitors the cellular sulfur
status and in response to low sulfur levels, turns on the expression of cys-3 which then activates the expression of the structural genes. The scon-2 gene is hypothesized to function as the communication link between scon-1 and cys-3 (Paietta 1990).

The wide metabolic versatility exhibited by filamentous fungi like Neurospora to use different nitrogen sources makes the nitrogen circuit one of the most intensely studied in terms of general regulation. When Neurospora is starved for a favored nitrogen source such as glutamine, glutamate or ammonia, it will resort to the metabolism of a variety of secondary compounds to meet its nitrogen requirement (Marzluf 1981). These secondary nitrogen sources include nitrate, nitrite, purines, urea, many amino acids, and even proteins. Indeed, these secondary compounds form a highly diverse group; yet, their assimilation and metabolism into utilisable nitrogen is controlled by a single global circuit that consists of major and minor (pathway-specific) regulatory genes, a wide range of unlinked nitrogen structural genes and specific metabolic inducers and repressors.

According to the model for nitrogen regulation as shown in Figure 1, the absence of the primary nitrogen compounds derepresses the system and allows the major positive regulatory gene, nit-2, to turn on the expression of the appropriate unlinked nitrogen structural genes. Another unlinked regulatory gene, nmr-1, negatively acts within the circuit and represses synthesis of the enzymes. Moreover, most pathways within the nitrogen circuit require specific induction by a key metabolite along with derepression by the nit-2 gene for enzyme synthesis. In the particular case of nitrate assimilation, an additional pathway-specific positive regulatory gene, nit-4, mediates the induction by nitrate which with simultaneous derepression by nit-2, elicits expression of the structural
genes, *nit-3* and *nit-6*, which encode nitrate and nitrite reductases, respectively. Much of what is known or surmised about the regulatory mechanisms within the nitrogen circuit have come from extensive genetic and biochemical analyses of individual pathways. Thus, the relatively recent cloning and characterization of each regulatory gene and some representative nitrogen structural genes have greatly enhanced the molecular understanding of how they interact within the circuit.

Detailed genetic studies of *nit-2* null mutants have suggested that the *nit-2* gene encodes a *trans*-acting major positive regulatory protein as exhibited by pleiotropic loss of many nitrogen utilizing enzymes (Reinert and Marzluf 1975) and the suppression by *Ssu-1*, a tRNA suppressor gene, of an amber *nit-2* mutant (Perrine and Marzluf 1986). Its expression appears to be regulated, albeit not autogenously, as observed by a higher level of *nit-2* expression under derepressed conditions but no discernible difference in expression for wild-type or mutant *nit-2* strains grown under contrasting nitrogen conditions (Fu and Marzluf 1987a). The putative NIT-2 protein (Fu and Marzluf 1990a) possesses a single Cys$_2$/Cys$_2$ zinc finger domain which shows significant homology to the mammalian GATA zinc finger domain (Tsai *et al.* 1989) and two short acidic and basic regions that may function as activation regions. Deletions of up to 21% of the carboxyl terminus has no effect on NIT-2 function, and *in vitro* DNA-binding and footprinting studies have demonstrated that an overexpressed *E. coli* partial NIT-2 protein containing essentially the zinc finger domain has sequence-specific DNA-binding properties (Fu and Marzluf 1990b). More specifically, these studies have shown that the NIT-2 zinc finger domain recognizes a short core sequence, TAGATA, that is highly homologous to the
mammalian GATA recognition sequence, AGATA. Although yet unclear, the presence of a central unprotected region within the NIT-2 binding sites for some nitrogen structural genes has been hypothesized to suggest a bipartite nature of NIT-2 (Fu and Marzluf 1990b).

Another regulatory gene, *nmr-1*, has been speculated to be a possible major negative control gene of the nitrogen circuit. All mutants of *nmr-1* so far have been shown to be constitutive, resulting in a loss of ammonia catabolic repression for a number of the nitrogen utilizing enzymes even under fully repressed conditions (Premakumar *et al.* 1980; Dunn-Coleman *et al.* 1981). However, the negative regulatory effect observed for *nmr-1* seems to not be as a repressor of *nit-2* expression (Fu and Marzluf 1988b) though it does decrease expression of representative structural genes (Fu and Marzluf 1987b). Molecular studies of the *nmr-1* gene has revealed that its putative protein sequence has no known DNA-binding motifs and deletion of up to 16% of its carboxyl terminus still permits negative function (Young *et al.* 1990). Further functional analyses of *in vitro* generated and conventional *nmr-1* mutants have not identified a single domain that is responsible for the observed negative activity by the protein, suggesting that large, perhaps overlapping, domains are involved (Jarai and Marzluf 1990; Young and Marzluf 1991). It appears that *nmr-1* and *nit-2* work together to regulate the nitrogen circuit although the manner in which they interact is not understood. It has been postulated that the *nmr-1* regulatory protein may bind to and inhibit NIT-2 protein activity in the presence of glutamine, possibly by interfering with the functional activation or DNA-binding ability of NIT-2 (Dunn-Coleman *et al.* 1981; DeBusk and
In addition to the major regulatory genes, *nit-2* and *nmr-1*, which are important for controlling the expression of a variety of unlinked structural genes in the nitrogen circuit, repression and induction by key substrates for a specific pathway are also vital for the production of a host of secondary nitrogen utilizing enzymes. Since so many different unlinked nitrogen structural genes are under the control of the nitrogen circuit, this additional regulatory mechanism allows for the expression and synthesis of only those enzymes necessary for metabolizing the secondary nitrogen source available at the time. In *Neurospora*, many of the nitrogen pathways within the circuit require their own specific metabolic inducer (Marzluf 1981); however, the major metabolic repressor for the entire cycle appears to be intracellular glutamine (Premakumar *et al.* 1979). When glutamine levels are adequate, expression of a number of structural genes of secondary nitrogen acquisition pathways, such as those for uricase and nitrate reductase, is repressed. Therefore, repression appears to occur at the transcriptional level (Premakumar *et al.* 1979; Wang and Marzluf 1979) although the mechanism by which it occurs is not well understood. From genetic studies of glutamine synthetase mutants, Dunn-Coleman and Garrett (1980) have proposed a complicated model in which the presence or absence of ammonia has a conformational effect on the glutamine synthetase protein, such that in one form it represses *nit-2* expression.

In contrast to the global repression exerted by glutamine, many enzymes of the nitrogen circuit are inducible by a substrate or key intermediary of the pathway in which they are found. In the purine catabolic pathway, for instance, four of seven structural
genes require both specific induction by uric acid and derepression by nit-2 for enzyme synthesis (Reinert and Marzluf 1975). A representative inducible gene of this pathway is the alc gene (Lee et al. 1990a) which encodes the allantoicase enzyme. In vitro footprinting studies of the alc promoter have demonstrated that multiple sequence-specific binding sites exist for NIT-2 but not for NMR-1 (Lee et al. 1990b), suggesting that NIT-2 acts as a DNA-binding transcriptional activator of alc. In addition, it has been postulated from Northern analyses that yet another pathway-specific positive regulatory gene analogous to the purine catabolic minor control gene, uaY, of Aspergillus nidulans (Scazzocchio et al. 1982) may be involved in mediating specific induction of alc. That is, under derepressed-induced conditions, ALC enzyme activity is elevated but the mRNA level does not differ significantly from that under repressed conditions (Lee et al. 1990a).

Owing to its agricultural importance and interesting mode of regulation, one of the best biochemically and genetically investigated pathways of the Neurospora nitrogen circuit is that for nitrate assimilation. The nitrate assimilatory pathway is comprised of two enzymes which catalyze the successive reduction of nitrate to utilizable ammonia via nitrite. The unlinked structural genes, nit-3 and nit-6, encode nitrate reductase and nitrite reductase, respectively. Their expression requires the coordinated action of both the major positive regulatory gene, nit-2, and an additional pathway-specific positive control gene, nit-4, as well as the presence of nitrate inducer. The NIT-2 protein has been shown in vitro to bind to three sequence-specific sites in the nit-3 promoter region (Fu and Marzluf 1990b), strongly suggesting that NIT-2 acts as a transcriptional activator of nit-3 expression. As with the alc gene, no DNA-binding properties were observed for
*nmr-1* with the *nit-3* promoter (Young, unpublished results), and other regulatory mechanisms such as protein-protein interactions may instead be involved.

In contrast to the pleiotropic effect of *nit-2*, the unlinked *nit-4* regulatory gene specifically controls the syntheses of nitrate and nitrite reductases; *nit-4* mutants do not seem to affect the expression of other nitrogen structural genes (Tomsett and Garrett 1980). Although the mechanism by which *nit-4* interacts within the pathway is uncertain, *nit-4* has been shown to act independently of *nit-2* (Fu et al. 1989a) and is proposed to mediate its effect through different binding sites upstream of the structural genes, thereby turning on the expressions of *nit-3* and *nit-6* in the presence of nitrate (Tomsett and Garrett 1980). As a putative transcriptional activator, the *nit-4* protein is postulated to have at least two functionally distinct domains. The recent cloning (Fu et al. 1989a) and molecular characterization of the *nit-4* gene have shown that it may, indeed, encode a protein with a putative Cys$_6$-type zinc finger DNA-binding domain in addition to a glutamine-rich region which may function in transactivation (Yuan et al. 1991).

Besides the regulatory effects of *nit-2* and *nit-4* in the presence of a nitrate inducer on *nit-3* and *nit-6* expression, biochemical and genetic studies with certain *nit-3* mutations which affect the structural integrity of nitrate reductase have shown constitutive expression even under uninduced conditions. These studies suggest that nitrate reductase may autogenously regulate its own synthesis (Tomsett and Garrett 1981; Fu and Marzluf 1988a) as well as that of nitrite reductase. The proposed model for the autoregulation of nitrate reductase in its simplest form postulates that nitrate reductase binds to the *nit-4* regulatory protein and inhibits the activation of the nitrate reductase and nitrite reductase
structural genes, possibly by either preventing the nit-4 protein from entering the nucleus or modifying its activation property. In the presence of the inducer (nitrate), however, nitrate reductase binds nitrate, and releases the nit-4 protein which can then turn on the nitrate and nitrite reductase structural genes. Thus, nitrate reductase is presumed to have both a catalytic and a regulatory function.

In comparison to that of Neurospora, the genetic organization of the nitrate assimilatory pathway of another ascomycete, Aspergillus nidulans, consists of three tightly linked structural genes that are clustered together and known thus far to be regulated by two unlinked control genes named areA (Arst et al. 1979; Johnstone et al. 1989) and nirA (Cove 1979). Analogous in function to the nit-2 and nit-4 regulatory genes, respectively, of Neurospora, the areA gene turns on the expression of a variety of nitrogen structural genes including the crmA, niaD and niiA genes of the nitrate cluster (Arst and Cove 1973), whereas nirA only specifies the expressions of the genes for nitrate and nitrite reductases; i.e., niaD and niiA, respectively. Moreover, each gene is expressed as individual mRNA transcripts, and regulation of expression appears to occur at the level of transcription (Arst et al. 1979; Johnstone et al. 1989). Nitrate reductase is also assumed to be autoregulatory and in fact, the autogenous regulation model for nitrate reductase synthesis was originally hypothesized from formal genetic studies in Aspergillus (Cove and Pateman 1969).

Evidence that regulatory genes and their sites of action are evolutionarily conserved between the two divergent fungal species comes from complementation studies in which the transformed nit-2 gene was able to restore areA mutants to a wild-type
phenotype, though weak repression by nitrogen was observed in the transformants (Davis and Hynes 1987). Furthermore, in vitro studies have shown that the NIT-2 protein recognizes and binds to several specific sequences in the promoter regions of niaD and niiA (Fu and Marzluf 1990b). Similarly, heterologous expression and regulation by nit-4 have been found in nirA mutants of Aspergillus though full nitrogen repression once again was not seen (Hawker et al. 1991). No evidence for a negative-acting regulatory gene analogous to nmr-1 has yet been obtained in Aspergillus (Wiame et al. 1985) although Southern analysis of amplified Aspergillus genomic DNA has identified regions that could hybridize with an nmr-1 probe (Young and Marzluf 1991). However, the characterizations of both null and constitutive areA mutants seem to imply that the areA gene may function negatively as well as positively in regulation (Arst and Cove 1973).

The relatively recent developments in genetic engineering have permitted extensive and rapid molecular analysis of nitrate assimilation in filamentous fungi like Aspergillus and Neurospora as well as in other fungi, plants and algae. On the other hand, the biochemistry of the entire nitrate assimilatory process is not as well-characterized in most nitrate-utilizing organisms. Instead, the primary emphasis in fungi and plants has been on understanding the catalytic efficiency and regulation of nitrate reductase not only since it catalyzes the very important rate-limiting step in the nitrogen acquisition process but also because it is frequently considered in plants to be the restricting factor of growth and development and thus, can potentially be improved for enhanced agricultural productivity.
The complete reduction of nitrate to ammonia is an energetically expensive, two-step process that requires a total of eight electrons. In the first step, which is catalyzed by the enzyme nitrate reductase, two electrons are transferred from NADPH (or NADH) to nitrate, producing nitrite. Thereafter, nitrite reductase catalyzes the conversion of nitrite to ammonia via a six-electron transfer. In plants and algae, the latter step is known to be coupled to the photosynthetic electron transport chain through reduced ferredoxin which acts as the physiological reducing agent (Solomonson and Barber 1990).

Of the three isoforms of assimilatory nitrate reductase, which vary only in the specificity of the electron donor (Campbell 1989; Garrett and Amy 1978; Solomonson and Barber 1990), fungi like Neurospora and Aspergillus prefer the NADPH-form of nitrate reductase (E.C. 1.6.6.3) while higher plants are predominantly specific for the NADH-type (E.C. 1.6.6.1). A bispecific NAD(P)H:nitrate reductase (E.C. 1.6.6.2) isoform occurs in eucaryotic algae and in the roots of some plants such as barley, rice and maize. In most organisms, nitrate reductase is coded for by a single gene. However, two functional nitrate reductase genes have been genetically determined in barley and postulated to specify different isoforms of the protein (Caboche and Rouzé 1990).

With the exception of the algal Chlorella nitrate reductase, which is a homotetramer (Howard and Solomonson 1982), plant and fungal nitrate reductase proteins that have been characterized exist as homodimers with each subunit comprised of three functionally distinct and physically separate co-factor binding domains that act as individual redox centers. The flavin, heme and molybdenum-pterin cofactor (MoCo)
prosthetic groups are present in a 1:1:1 ratio per monomer and in the sequential order given, catalyze nitrate reduction (Redinbaugh and Campbell 1985; Solomonson and Barber 1990). The flavin cofactor is FAD (Evans and Nason 1953) while the heme cofactor is of the b5-type cytochromes (Garrett and Nason 1967). The molybdenum cofactor is a pterin protein that is necessary for holoenzyme assembly as well as catalysis (Pan and Nason 1978). The same cofactor is also present in other molybdenum cofactor-containing proteins such as xanthine dehydrogenase since a Neurospora nitrate reductase mutant deficient in the molybdenum cofactor can be complemented to functionality in vitro with extracts of different molybdenum-containing enzymes (Nason et al. 1971). Intragenic complementation was also observed in plant and algal nitrate reductases (Caboche and Rouzè 1990) and in Aspergillus (Garrett and Cove 1976) apoenzyme mutants. The reported molecular mass for the nitrate reductase subunit in a variety of organisms differs widely, ranging from approximately 100 to 145 kDa with the average mass at about 110 kDa (Solomonson and Barber 1990). For Neurospora nitrate reductase, the subunit molecular mass has been estimated to be around 114 kDa (Bahns and Garrett 1980). In addition, Neurospora nitrate reductase is highly reactive with a turnover number of 18,000 although it has been determined to be present at a very low concentration (1.7 pmoles enzyme/mg protein; Garrett and Amy 1978).

Four additional loci in Neurospora, besides nit-3, have been shown genetically to specify the synthesis of nitrate reductase (Tomsett and Garrett 1980). These loci termed nit-1, nit-7, nit-8 and nit-9 are known to be necessary for the synthesis and assembly of the molybdenum cofactor. Similarly, six different loci known as the cnx
genes have also been shown to be important in molybdenum cofactor biosynthesis in *Aspergillus* (Cove and Pateman 1963) and tobacco (Muller and Mendel 1989). One locus, *cnxA*, has, furthermore, been proposed to be involved in complexing the molybdenum metal ion with the organic pterin cofactor.

In addition to the reduction of nitrate, all eucaryotic nitrate reductases can catalyze other partial activities *in vitro* by utilizing artificial electron donors and acceptors for assay of one or two of the prosthetic groups. That is, with NADPH (or NADH) as the electron donor, artificial electron acceptors such as ferricyanide or cytochrome c may be used to assay for partial activity in the heme- and/or flavin-binding domains (Solomonson and Barber 1990). Moreover, the reductase activity of the enzyme allows the use of artificial electron donors like reduced methyl viologen, FMNH₂ or bromphenol blue (Schrader *et al.* 1968; Campbell 1986; Hoarau *et al.* 1986) for assay of the associated partial activities in the heme- and/or molybdenum-cofactor binding domains. Therefore, it is possible to assay for the functionality of a single domain independently of another.

Structural and functional studies using limited proteolysis and partial activity assays of spinach (Kubo *et al.* 1988) and *Chlorella* (Solomonson *et al.* 1986, 1987) nitrate reductases have shown that the prosthetic groups are, indeed, held in discrete domains which are covalently linked together by protease-sensitive hinge regions. From these studies it has been proposed that the molybdenum-cofactor domain may be significant in dimer formation possibly via a stable intermolecular disulfide bond between the two domains. Biochemical studies with purified *Neurospora* nitrate reductase protein
(Garrett and Nason 1969) and immunoaffinity-purified corn and squash nitrate reductase proteins (Hyde et al. 1989) have also implied that a reactive thiol group in the flavin-binding domain is directly involved in nitrate reduction. Recently, however, a partial tobacco nitrate reductase expressed in *Saccharomyces cerevisiae* (which has no endogenous nitrate reductase), still showed dimer formation although the protein lacked the MoCo domain. This has further suggested that the MoCo domain is more important in stability, and is not necessarily required for dimer formation (Truong et al. 1991).

The reductive reaction catalyzed by nitrate reductase is basically irreversible \( (K_{eq}=10^{10}) \) with the nitrite and NADP\(^+\) (or NAD\(^+\)) products acting as inhibitors. The kinetics of nitrate reductase, however, are not simple, and a variety of steady-state kinetic mechanisms have been proposed for nitrate reductase from different sources. Although no kinetic studies have been reported for *Neurospora* nitrate reductase, a random-order rapid-equilibrium mechanism has been postulated for the enzyme from *Aspergillus* (McDonald and Coddington 1974). Similarly, *Chlorella* nitrate reductase has been speculated to have a rapid-equilibrium random bi bi kinetic mechanism (Howard and Solomonson 1981). In contrast, a two-site ping pong mechanism, which is a composite of the standard ping pong and random order kinetic mechanisms and involves physical transfer of the substrate from one redox site to the next, has been proposed for the nitrate reductases of the fungus, *Penicillium chrysogenum* (Renosto et al. 1981), and of corn and squash (Campbell and Smarrelli 1978). Moreover, kinetic studies of the partial activities of *Chlorella* nitrate reductase have demonstrated that the rate-limiting step appears to be the transfer of electrons from heme to molybdenum (Howard and Solomonson 1981).
In spinach nitrate reductase, intramolecular electron transfer was not rate limiting (Barber and Notton 1990).

A variety of environmental factors have been found to affect the synthesis of nitrate reductase. For the most part, the primary mode of regulation in fungi and plants appears to depend upon the kind of nitrogen source available whether it be induction by nitrate or repression by ammonia. Two exceptions are the nitrate reductase proteins of *Chlorella vulgaris* (Zeiler and Solomonson 1989) and the yeast, *Candida nitratophila* (Cannons and Hipkin 1987), which seem to require only ammonia derepression for synthesis. In algae and higher plants, light also plays a major role in nitrate reductase regulation. Squash leaves grown in the absence of light have demonstrated that nitrate reductase expression is under phytochrome control (Rajasekhar *et al.* 1988). Cyclic light-dark studies with tomato and tobacco leaves and roots revealed that light enhances nitrate-induced expression of the protein although it causes a rapid decrease in mRNA content, suggesting that nitrate reductase expression is controlled by circadian rhythm (Galangau *et al.* 1989; Deng *et al.* 1990). Additional light-dark studies with functional and nonfunctional chloroplasts in corn, furthermore, showed that nitrate reductase activity but not its mRNA level, was lower in nonfunctional chloroplasts (Gowri and Campbell 1989). The regulation of nitrate expression by nitrate, light and circadian rhythm seems to occur at the transcriptional level as shown from studies with transgenic tobacco plants. In addition, the regulatory effect of light on nitrate reductase protein accumulation in these plants implies that expression may also be translationally or posttranslationally controlled (Vincentz and Caboche 1991). The effect of plant hormones on nitrate
reductase synthesis has not been extensively studied though in one species of plants, cytokinins and ethylene affected induction of nitrate reductase activity (Schmerder and Borris 1986).

Because nitrate reductase is a highly labile protein, the main problem encountered in biochemical studies has been the difficulty of purifying the protein to homogeneity. However, the relatively recent cloning and characterization of nitrate reductase genes from a number of nitrate-utilizing sources including maize, rice, barley, tomato, tobacco, squash, Arabidopsis, Chlamydomonas, Neurospora and Aspergillus have allowed for rapid progress toward understanding the structural and regulatory aspects of nitrate reductase at the molecular level. Currently, no regulatory genes analogous to nit-2, nmr-1 and nit-4 of Neurospora have been found in plants. However, recent in vitro binding studies by Jarai et al. (1992) showed that a NIT-2 zinc finger domain/β-gal fusion protein was able to specifically bind, albeit weakly, to two regions upstream of the nitrate reductase gene from tomato. Each of these regions were observed to contain the core GATA recognition sequence, suggesting that a regulatory protein homologous to NIT-2 may exist in higher plants.

In order to examine the function of genes manipulated in vitro, using molecular biological methods, a reliable system of incorporating exogenous DNA into an organism’s genome is necessary. A prerequisite for DNA-mediated transformation studies is that the cells must be made competent for DNA uptake. The transformation techniques used for filamentous fungi like Neurospora are based on methods that were originally developed for the yeast, Saccharomyces cerevisiae (Hutchinson and Hartwell
Although the popular procedure for making competent cells, or spheroplasts, in Neurospora is still by partially digesting the cell wall enzymatically, alternate protocols have also been employed successfully and include the use of alkali metal ions (e.g. lithium) at high concentrations to induce intact cells to take in DNA (Dhawale et al. 1984) and electroporation of germinated conidia (Chakraborty and Kapoor, 1990). Particle bombardment (Klein 1984) and partial cell breakage by blending with glass beads (Costanzo and Fox 1988) have been tried in Saccharomyces but not in filamentous fungi.

Early work on germinated conidia or hyphae treated with either of two cell wall-digesting enzyme preparations, snail gut Gluculase (Hinnen et al. 1978; Kinsey et al. 1984) or Zymolyase (Hsiao and Carbon 1979) of microbiological origins, resulted in varying degrees of cell transformability owing largely to problems associated with degradation of the cell wall. The use of Novozym 234, a commercial mixture of hydrolytic enzymes secreted by the filamentous fungus, Trichoderma harzianum, has been found to be the most successful in yielding highly transformable spheroplasts since hyphae appear to be extremely vulnerable to this enzyme preparation (Akins and Lambowitz 1985). The transformation procedure itself is relatively simple. After partial or complete digestion of the fungal cell walls, exogenous DNA with calcium ions and heparin are introduced and the spheroplasts are induced to fuse, or regenerate, in the presence of polyethylene glycol (Vollmer and Yanofsky 1985). Transformants with the desired phenotype are selected on osmotically stable regeneration medium. Sorbitol or magnesium sulfate is frequently used as an osmotic stabilizer once the fungal cells have been made competent.
Two methods generally used for the selection of positive transformants against a nontransformant background are either complementation of auxotrophic mutants or the use of a dominant selectable marker. Complementation of an auxotroph by an introduced gene was initially shown to occur in *Neurospora* by Mishra and Tatum (1973) and later confirmed by Case *et al.* (1979) to be a chromosomal integration event using the cloned *qa-2* gene. A major disadvantage of this method, however, is that it is highly dependent upon the availability of the appropriate auxotrophic strains. For well-studied fungi like *Neurospora*, this is usually not a problem but with poorly characterized organisms of medical, agricultural or industrial importance, this method is often not feasible.

An alternative to auxotrophic complementation and a more versatile method is the use of a dominant selectable marker that does not require a corresponding mutant strain since these dominant selectable markers often confer resistance to antibiotics in transformants. Although selection by dominant marker is generally species-specific and dependent upon the susceptibility of the transforming strain used, they have been very useful in cotransformation experiments when it is difficult or impossible to directly select for the transforming gene of interest. The most commonly employed dominant selectable marker in *Neurospora* is the mutant β-tubulin gene (Orbach *et al.* 1986) which confers resistance to benomyl in transformants because the antibiotic fails to bind to the mutant protein. Another dominant selectable marker is the cloned bacterial hygromycin-resistant phosphotransferase gene (Staben *et al.* 1989). Unlike the β-tubulin gene, no homologous phosphotransferase gene is endogenously present in fungi, and thus, allows for the simple transformation of less investigated but industrially important fungi.
Although autonomously replicating plasmids have been reported (Stohl and Lambowitz 1983; Grant et al. 1984), transformation of Neurospora (and other filamentous fungi) is presently best achieved by genomic integration, resulting in three possible fates for the introduced DNA. Homologous recombination, or targeting, may occur through simple gene conversions or double crossover events as well as by single crossovers that could lead to multiple copies in tandem. Homologous integration appears to be a rare event in Neurospora and seems to largely depend upon the transforming DNA involved. For example, about a 50% homologous recombination frequency was originally observed with the qa-2 gene (Case et al. 1979) but later appears to be about 5-10%, while with the glutamate dehydrogenase gene, am+, the frequency was close to 33%. A case of gene disruption in Neurospora using linear DNA and lithium prepared spheroplasts was found to attain only about a 10% homologous recombination frequency (Paietta and Marzluf 1985). The more prevalent form of integration in Neurospora is via heterologous integration such that one or more plasmid copies are inserted throughout the genome in an ectopic manner. Moreover, integration sites not only differ from one transformant to another (Case et al. 1979; Paietta and Marzluf 1985), but also may occur exclusively in a single nucleus of a heterocaryotic cell, thereby often complicating interpretation of transformation assay data.

Despite the difficulties encountered with fungal transformations, the technique has been extremely valuable in studying the basic mechanisms involved in growth, development and metabolism of fungi. In fact, many Neurospora genes, including those of the nitrogen circuit, have been cloned by an efficient and rapid sib selection procedure
which essentially utilizes genetic complementation of auxotrophic mutants (Akins and Lambowitz 1985). Furthermore, the development of heterologous transformation systems has aided in the identification of analogous genes between different fungal species (Unkles 1989). The use of transformation methods to genetically engineer industrially relevant fungi for enhanced product production is certainly of biotechnological significance (Timberlake and Marshall 1989). In fact, the fungal nitrate assimilatory pathway and nitrate reductase, especially, are of particular industrial interest for the development of heterologous expression/secretion systems due to, among other things, nitrate reductase's highly regulated, and thus tractable, nature (Unkles 1989).

Since the biochemistry and genetics are well-established for nitrate reductase, Neurospora is amenable for the study of the structure and function of the enzyme. In addition, the fact that partial activities of nitrate reductase domains can be functionally assayed independently of each other is a valuable means by which to study structure and function of a domain. Yet, in order to gain an even better comprehension of the regulation of the nitrate assimilatory pathway and nitrate reductase, molecular characterization of the nit-3 gene is imperative. Therefore, most of the studies on structure-function relationships depend upon genetic engineering of the nitrate reductase gene.

My research involves the molecular analysis of the nit-3 gene in Neurospora and the study of one of its cofactor-binding domains, the heme domain, using genetic, biochemical and molecular biological methods. The complete nit-3 nucleotide and translated protein sequences, its transcript size and binding domains for the prosthetic
groups in the protein have been determined. The regulation of nit-3 has also been examined in terms of the kinetics of mRNA synthesis and turnover under contrasting nitrogen conditions. Based on its relatively small and manageable size as well as its high homology to a mammalian cytochrome b$_5$ protein (whose crystal structure is known; Mathews et al. 1972), the heme domain was analyzed and key amino acid residues were identified using immunological, biochemical and genetic techniques. I have also expressed the heme domain in E. coli and used the purified protein for specific antibody production. A suitable host strain to analyze manipulated nit-3 genes via transformation would contain a deleted or severely damaged nit-3 gene. Since such a host strain was not available, I employed the recently described RIP (repeat-induced mutation) phenomenon (Selker et al. 1987) to prepare a valuable nit-3 mutant host strain. The work that is presented here provides a firm foundation from which further molecular and cellular studies on the nit-3 gene, nitrate reductase, and, in essence, the nitrogen circuit can be undertaken in Neurospora crassa.
CHAPTER II
EXPERIMENTAL PROCEDURES

*Neurospora crassa* strains and growth conditions

The *Neurospora crassa* wild-type strains, 74OR23-1A (74A) and 74OR23-1a (74a), and nit-3 mutant strains, 14789A and 14789a, were obtained from the Fungal Genetic Stock Center (Kansas City, KS). *N. crassa nit-3* mutant strains V1M16 and V1M4 (Tomsett and Garrett 1981) were kindly provided by Dr. R. Garrett of the University of Virginia. The nit-3 amber mutant strains and the corresponding mutant suppressor strains were previously isolated in this laboratory (Perrine and Marzluf 1986).

Routine genetic manipulations, stock maintenance and growth conditions were performed as described by Davis and deSerres (1970). The growth medium consisted of 2% sucrose, 0.01 μg/ml biotin and 1X Vogel’s minimal medium minus nitrogen. As the sole nitrogen source, either 20mM L-glutamine or 20 mM potassium nitrate was used and corresponded to nitrogen repressed conditions or nitrogen derepressed, nitrate induced conditions, respectively. Mycelia in liquid cultures were grown at 30°C under nitrogen-repressed conditions for a minimum of 15 hours with shaking at 150-180 rpm, harvested, rinsed with water and quickly frozen in liquid nitrogen. When induction was desired, the mycelia were harvested, washed several times with water and transferred to nitrate-
inducing media for an additional three hours of growth. Chlorate medium, which was routinely used to select for the \textit{nit-3} mutant phenotype, is 1X Vogel's minimal medium without nitrogen containing 5 mM urea and 100 mM potassium chlorate.

\textbf{Transformation of \textit{Neurospora crassa}}

The generation of \textit{Neurospora crassa} spheroplasts using Novozym 234 (Novo Industrials, Bagsvaerd, Denmark) and transformation of the spheroplasts were carried out basically as reported elsewhere (Akins and Lambowitz 1985). High spheroplasting efficiency and good viability were best obtained when the conidial cell walls were digested for 30 minutes with 1.3 mg/mL of Novozym 234 at 30°C with gentle agitation at 100 rpm. In all transformation experiments, 5 mL of top agar (Chemiack \textit{et al.} 1991) that had previously been equilibrated to 45-50°C were used to suspend the transformed spheroplasts. The top agar consisted of 500 mM MgSO$_4$, 1X FGS (0.05% fructose, 0.05% glucose, 2% sorbose), 0.6% agar noble (Difco, Detroit, MI), biotin and 1X Vogel's minimal medium \textit{sans} nitrogen and containing 20 mM of either L-glutamine or KNO$_3$ depending upon the desired selection. A 1 M MgSO$_4$ stock solution and 10X sugar stock solution were sterilized separately and cooled to 65°C before their addition to the rest of the agar mixture to give the final concentrations. The top agar was overlaid onto bottom agar plates which had an identical composition as the top agar except that the agar noble concentration was 1.5% and no MgSO$_4$ was included. When selection for benomyl or hygromycin resistance was desired, 0.5 \textmu g/mL benomyl (0.25 mg/ml stock solution in ethanol) or 0.2 \textmu g/mL filter-sterilized hygromycin B (Sigma Chemical Co., St. Louis, MO) was added to the cooled (65°C) bottom agar. Due to the
instability of these antibiotics, these plates were used immediately after they were prepared. All transformation assays included 25 \( \mu \)g of heparin. Whenever possible, DNA purified by CsCl centrifugation was used; in those cases in which mini-prepped DNA was used, 4 \( \mu \)g of spermidine pH 7, was mixed with the DNA prior to addition of the spheroplasts. Cotransformation assays were performed by combining a \( \text{nir-3} \) plasmid with a vector carrying dominant selectable marker at a ratio of 3:1 and 5:1 (w/w). The selectable markers used were either the benomyl-resistant gene on plasmid \( pSV50 \) (Vollmer and Yanofsky, 1986), or the hygromycin resistance gene on \( pCSN44 \) (Staben et al. 1989).

**Isolation of *Neurospora* homocaryons**

*Neurospora* homocaryons were isolated by either serial transfer under selective conditions (deSerres and Davis 1970) or from microconidia purified by passage through a Millipore Durapore Millex-SV 0.5 micron filter (Millipore Products Division, Bedford, MA) according to the method of Ebbole and Sachs (1990).

**Isolation of *Neurospora crassa* genomic DNA and poly(A)\(^+\) mRNA**

For large-scale DNA preparations and PCR analysis, *Neurospora crassa* genomic DNA was isolated using a modified method of Metzenberg and Baisch (1981). For small DNA preparations for restriction digestions and Southern analysis, a slight variation of the rapid mini-prep procedure of Leach et al. (1986) was followed. In the latter method, 10 mL of sterile Vogel's minimal media in a 18x150 mm culture tube were inoculated with a loopful of 7- to 10-day-old conidia, vortexed briefly to suspend the conidia and then shaken at 180 rpm, 30°C for 20-24 hours. The mycelia were harvested on
Whatman 1MM filter paper (Whatman International Ltd., Maidstone, UK) and immediately ground in a mortar and pestle with liquid nitrogen. The homogenate was then suspended in LETS buffer (0.1 M LiCl, 10mM EDTA, pH 8, 10 mM Tris-Cl, pH 8, 0.5% SDS), transferred to a 1.5 mL Eppendorf tube and vortexed at high speed for 2 minutes. Subsequent steps were exactly as described (Leach et al. 1986). The pellet obtained after ethanol precipitation was resuspended in 50-75 μL TE buffer and stored at -20°C. For restriction digestions, 15 μL of the genomic DNA were digested for at least 8 hours at 37°C with 20 U of enzyme in a total volume of 150 μL. The digested DNA samples were then precipitated with 1/10 volume of 3M NaOAc, pH 5.2, and the pellets resuspended in 50 μL TE buffer. After the addition of 10 μL of 6X sample loading buffer that contained 2 mg/ml DNase-free RNAse, the digests were analyzed by agarose gel electrophoresis (Sambrook et al. 1989).

Total RNA was isolated by the method of Reinert et al. (1981), and poly(A)+ mRNA was obtained by oligo(dT) chromatography (Aviv and Leder 1972). Two volumes of ethanol with NaOAc pH 5.2 (0.3M final concentration) were added to the collected mRNA solution, and the RNA was stored as an ethanol suspension at -70°C. Prior to use, the mRNA was pelleted at 4°C, very briefly vacuum-dried and resuspended in 25 μL of TES buffer. Approximately 10-20 μg of poly (A)+ mRNA were used for Northern analysis.

**Transformation of E. coli**

*E. coli* competent cells were prepared using the calcium chloride procedure and transformed by the method of Mandel and Higa (1970). Propagation of the plasmid was
generally with the *E. coli* strain, *XL1-blue* (Stratagene, LaJolla, CA), unless otherwise specified.

**Isolation of plasmid DNA**

Three methods were used to isolate plasmid DNA. For most experiments, plasmid DNA was isolated by the small-scale method of Birnboim and Doly (1979). However, when it was necessary to screen a large number of transformants for a desired subclone, the "lyse and load" method (Sambrook *et al.* 1989) was preferred. In this method, single colonies were individually picked with sterile toothpicks, suspended in 30 μL STE buffer and extracted with an equal volume of phenol:chloroform. The aqueous phase was then mixed with RNAse-containing loading buffer (2 mg/mL DNase-free RNAse, 30% glycerol, 0.25% bromphenol blue, 0.2% xylene cyanol, 10 mM EDTA) and analyzed by agarose gel electrophoresis. For comparison, undigested vector was used as a marker.

Large-scale preparations of plasmid DNA were performed basically as prescribed by Sambrook *et al.* (1989) and Ausubel *et al.* (1989). The only deviation from the published protocol was that once the DNA band was pulled from the cesium chloride gradient, the volume was noted and diluted 1:4 with TE buffer after several rounds of extraction with 5 mL butanol to remove the ethidium bromide. The DNA was precipitated with two volumes of ethanol and 3 M NaOAc, pH 5.2 by keeping the ethanol suspension on ice for at least 30 minutes, after which the DNA was pelleted by centrifugation for 10 minutes at 10K rpm at room temperature. The DNA was then resuspended in 100-150 μL of TE buffer. DNA concentrations were determined by
measuring the optical density at 260 nm on a Gilford 250 spectrophotometer.

Isolation of single-stranded DNA and site-directed mutagenesis

For sequencing experiments, it was often necessary to rapidly isolate a number of single-stranded DNAs. In these cases, good quality ssDNA was obtained by the method of Katayama (1990).

Single-stranded DNA for mutagenesis experiments was isolated by following the protocol included with the Bio-Rad Muta-gene kit (Richmond, CA) with slight modifications. The best quality and yield of ssDNA were obtained when the multiplicity of infection was 1:200 (dut- ung- E. coli strain CJ236 to helper phage VCS-M13), and, prior to phenol extraction of the phage, the suspension was incubated with 0.5 mg Proteinase K (Boehringer Mannheim, Germany) in the presence of 0.1% SDS for 60 minutes at 37°C.

Site-directed mutagenesis was performed as described in the Bio-Rad Muta-gene kit (Richmond, CA) which employs the Kunkel method (1985). The use of the T4 Gene 32 protein (0.5 μg; Bio-Rad, Richmond, CA) enhanced the efficiency of the T4 polymerase activity in DNA synthesis. Whenever possible, a restriction site was either introduced or deleted at or near the site to be mutagenized for ease of screening the E. coli transformants.

Isolation of DNA fragments

DNA fragments required for cloning and as probes were generally isolated according to the manufacturer's instructions for the GeneClean kit (Bio 101, LaJolla, CA) or with the Unidirectional Analytical Electroeuter Model UEA (International
Biotechnologies, Inc., New Haven, CT). In some cases, a modified version of a protocol by Heery et al. (1990) was also used. In this method, a small hole was made in the bottom of a 0.5 mL Eppendorf tube, plugged with sterile glass wool and the entire tube placed within a larger 1.5 mL Eppendorf tube. The ethidium bromide-stained agarose gel slice which contained the desired DNA fragment was put in the smaller tube and spun at 6000 rpm, 25°C for 20 minutes after which the extent of DNA elution was checked by following the migration of the dye under UV light. To increase recovery of the DNA fragment, the gel slice was further incubated with 100 µL TE buffer at 37°C for one hour and then spun again. The eluants were combined and ethanol precipitated. The washed and vacuum-dried pellet was then resuspended in 25 µL TE buffer.

**Kinetic accumulation and stability of the nit-3 mRNA**

For the kinetic accumulation studies, samples of wild-type *N. crassa* mycelia were grown for a constant 18-hour period in nitrogen repressed (glutamine) media after which they were transferred to and grown under nitrogen derepressed-nitrate induced conditions for various times at 30°C with shaking. Growth was stopped by harvesting and quick freezing the mycelial pads in liquid nitrogen. For mRNA stability studies, mycelia samples were uniformly nitrogen derepressed, nitrate induced for four hours at 30°C with shaking, harvested and then transferred to nitrogen repressed conditions. Samples were harvested at varying times in the same manner as described above.

**Southern and Northern analyses**

Southern and Northern blots were carried out as described in Sambrook et al. (1989). Northern gels were composed of 1% agarose, 10 mM phosphate buffer, pH 7.4,
and 6.7% formaldehyde, and electrophoresis was carried out at 100V with constant buffer circulation. Nucleic acids were cross-linked onto nitrocellulose using the Stratagene UV Stratalinker (LaJolla, CA) set at automatic crosslink. For both Northern and Southern analyses, the prehybridization solution consisted of 0.25% dry milk, 0.5% SDS, 1X SSC and 50% formamide (Ultrapure Reagent; Bethesda Research Laboratories, Gaithersburg, MD). The hybridization solution was the same except 0.1% SDS was used and 10^6 cpm of denatured probe was added per 1 mL of solution. The probes were labelled using either the nick-translation or the random primer kit as instructed by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD) and eluted through a G-50 Sephadex (Pharmacia, Piscataway, NJ) spun column (Sambrook et al. 1989) to remove unincorporated isotope prior to radioactive measurements in an LKB 1209 liquid scintillation counter (Gaithersburg, MD). Filters were washed twice in 1X SSC and 0.1% SDS at room temperature for 15 minutes each time followed by two additional washes in 0.1X SSC and 0.1% SDS for 30 minutes at 65°C.

**Purification of synthetic oligonucleotide primers**

Oligonucleotide primers were synthesized on an Applied Biosystems model 380B DNA Synthesizer (Ohio State University Biochemical Instrument Center, Columbus, OH). The primers were deblocked by incubating for at least 6 hours at 55°C, cooled to room temperature and then carefully transferred to clean, DNAse-free 15 mL Evapomix tubes and vacuum-dried in an Evapomix (Buchler Instruments, Inc., Fort Lee, NJ). After evaporating the NH₄OH storage solution, the primers were washed with sterile water and vacuum-dried again. This step was repeated once. The deblocked primers
were then resuspended in 1 mL of TE buffer. Desalting of the primers was done by adsorption chromatography using Nensorb 20 columns (Dupont NEN Products, Boston MA) as instructed by the manufacturer. Approximately 200 μL of the initial eluant were collected, aliquoted into 50 μL samples, vacuum-dried and finally, resuspended in TE buffer. The concentration was determined by measuring the UV absorption at 260 nm on a Gilford 250 spectrophotometer. The deblocked or deblocked and desalted primers were stored at -20°C.

DNA sequence determination and analysis

Nested deletion subclones of \textit{pnit-3} (Fu and Marzluf 1987a) and \textit{pnit3b}, which consists of the entire 5'-end of the gene, were made by using exonuclease III and mung bean nuclease as previously described (Fu and Marzluf 1988a). Templates for sequencing were either double-stranded plasmids or single-stranded DNA. Three to five μg of double-stranded plasmid DNA were alkali-denatured prior to sequencing. DNA sequencing was via the dideoxynucleotide chain termination method (Sanger \textit{et al.} 1977) using the modified T7 polymerase, Sequenase (United States Biochemical Co., Cleveland, OH). Sequencing reactions were resolved on 6% polyacrylamide, 8M urea denaturing gels. The use of Taq polymerase, dITP in place of dGTP, and single-stranded templates facilitated the sequencing of highly compressed regions that were otherwise difficult to read. Oligonucleotide 17-mer primers which annealed to sequenced regions were used to complete sequence gaps between the nested deletion subclones. All DNA and amino acid sequence analyses including translation, predicted protein structure, and amino acid homology comparisons were done with the IBI-Pustell Sequence Analysis
software program, ver. 2.02.

cDNA library screen

A *N. crassa* cDNA library was constructed in λgt10 as earlier reported (Fu and Marzluf 1990a) and screened for *nit-3* cDNA clones according to the procedures of Sambrook *et al.* (1982). Probes were made from nick-translated plasmids that contained either most of the *nit-3* gene or the 5′-end of the gene. Several rounds of plaque purification were required to isolate *nit-3* cDNA-containing phage. Purified lambda DNA was digested with EcoRI which liberated the *nit-3* cDNA insert. After subcloning the cDNA fragments into pBluescript (Strategene, LaJolla, CA), the cDNA clones were sequenced as already described.

**S1 nuclease mapping and primer extension analysis**

S1 nuclease mapping was accomplished by the method of Berk and Sharp (1977) using 600U of S1 nuclease, 100 ng of primer, and 10 μg of poly (A)+ mRNA. To map the 5′-end of the *nit-3* transcript, a 1.2 kb *BamHI-KpnI* fragment was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim, Germany) and then end-labeled with polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD) and γ-32P ATP (Amersham, Arlington Heights, IL). An additional digestion with *XbaI* yielded a uniquely end-labeled 870 bp *BamHI-XbaI* probe. Likewise, a 1 kb *BglII-KpnI* fragment was used to map the 3′-end of the *nit-3* transcript and labeled at one end by filling in the *BglII* site with Klenow fragment in the presence of all nucleotides. Primer extension analysis was carried out with a 5′ end-labeled 17-mer oligonucleotide primer that hybridized to a region approximately 250 bp from the putative transcriptional start site.
Extension reactions were at 37°C for 60 minutes using 1000U of MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 10 μg of poly(A)* mRNA, and 100 ng of labeled primer. For both S1 nuclease mapping and primer extension experiments, the reaction products were electrophoresed on a 6% polyacrylamide denaturing gel and exposed overnight or longer to Kodak XAR film (Rochester, NY) at -70°C with an intensifying screen.

**Polymerase chain reaction and subcloning of fragments**

For the polymerase chain reaction (Saiki et al. 1988), 25-50 ng of genomic DNA, 1.5 mM MgCl₂, 1.5 mM of each dNTP, 250 ng of each primer and 1X reaction buffer (10 mM Tris pH 8.3, 50 mM KCl) in a 0.5 mL tube were brought up to a total volume of 50 μL with sterile water. After overlaying the mixture with an equal volume of mineral oil, it was heated for 5 minutes at 95°C to destroy any contaminating proteases that might be present and then quickly chilled on ice. To the aqueous layer, 2.5 U of Taq polymerase (Bethesda Research Laboratories, Gaithersburg, MD) was carefully added, and the sample was placed in an automated thermal cycler (Ericomp Inc, San Diego, CA) which was set with the following parameters: 94°C for 30 seconds, 45°C for 1 minute, 72°C for 3 minutes. After 30 cycles, an additional cycle at 94°C for 30 seconds, 45°C for 1 minute, and 72°C for 7 minutes was run to complete the amplification process. Once amplification was completed, the aqueous phase was carefully transferred to a new 1.5 mL tube, and a 5 μL aliquot was analyzed by agarose gel electrophoresis.
Amplified DNA was usually subcloned into the EcoRV site of pBluescript (KS-; Strategene, LaJolla, CA) by filling in the amplified DNA fragment ends with 2 U of T4 polymerase (Bethesda Research Laboratories, Gaithersburg, MD) in the presence of 0.4 mM of each dNTP and following the protocol of Sambrook et al. (1989).

**Protein expression in E. coli**

Expression in *E. coli* was accomplished with the T7 expression system (Studier et al. 1990) which utilizes both the pET expression vector series as well as a genetically engineered *E. coli* strain, BL21(DE3)pLysS, that has the T7 RNA polymerase gene integrated into its genome and under the control of a lacUV5 promoter. The pET vectors carry the highly efficient T7 RNA polymerase promoter for transcription of foreign genes. Thus, addition of IPTG induces T7 RNA polymerase which, subsequently, turns on the expression of the target DNA.

In order to express part of the *Neurospora* nitrate reductase protein in *E. coli*, a BamH1 linker (Bethesda Research Laboratories, Gaithersburg, MD) was blunt-end ligated into the SmaI site of a SmaI-Sty1 nit-3 subclone which contains the nucleotide sequence for the entire heme domain and some flanking sequence. The introduced BamH1 site and a unique BglII site within the nit-3 subclone were used to insert the nit-3 fragment into the BamH1 site of pET3c, resulting in the expression plasmid, pETNIT3S, which contained the cloned DNA fragment in the proper reading frame with respect to the start codon in the vector. In this way, the first 12 amino acids of the expressed protein would be encoded by plasmid sequences followed by 217 amino acids of *Neurospora* nitrate reductase.
The pETNI3S subclone was transformed into E. coli strain BL21(DE3)pLysS for expression. A single colony was picked and used to inoculate 5 mL of LB medium supplemented with ampicillin and chloramphenicol for overnight growth. To 10 mL of supplemented LB medium, 50 µL of overnight culture were added, and the cells were grown to an optical density (OD$_{590}$) of about 0.5. After saving a 1 mL aliquot, the culture was induced with 1 mM IPTG (final concentration) for 1 to 3 hours, and 1 mL samples were drawn every hour. Immediately following each sample collection, the cells were centrifuged for 1 minute at 14K rpm and room temperature, resusupended in 50 µL of SDS-loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 0.00125% (w/v) bromphenol blue, 2% SDS, 7.2 mM β-mercaptoethanol) and then stored at -70°C until all the samples had been collected. Thereafter, the samples were boiled for 5 minutes, quickly chilled on ice and spun briefly in a microcentrifuge. The supernatant (20 µL) was loaded onto a 12% discontinuous polyacrylamide gel in a vertical slab gel apparatus (Bio-Rad, Richmond, CA) and SDS-PAGE was carried out as reported elsewhere (Laemmli 1970) using the Amersham Rainbow™ marker proteins (Arlington Heights, IL) to estimate molecular weights. The gel was stained with Coomassie R-250 Brilliant blue dye (Sigma Chemical Co., St. Louis, MO) as described elsewhere (Sambrook et al. 1989). During the overnight destaining step, a foam stopper was used to absorb the dye.

**Protein purification and polyclonal antibody production**

For partial protein purification, a modified version of the procedure by Lin et al. (1987) was used. Unless otherwise stated, all of the purification steps were carried out at 4°C. In this method, a 500 mL transformed BL21(DE3)pLysS culture was grown and
induced with IPTG as described in the preceding section. After induction for 3 hours, 1 mL aliquots of uninduced and induced cells were set aside for SDS-PAGE to check the extent of expression. The remaining cells were harvested by centrifugation at 4000xg for 5 minutes and then resuspended in 2 mL lysis buffer (50mM Tris, pH 8, 2 mM EDTA, 20 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF)) per 100 mL of culture. Lysozyme and sodium deoxylcholate were added to final concentrations of 1 mg/mL and 0.04%, respectively. Complete lysis was obtained by sonification (Sonifier Cell Disruptor, SmithKline Corp.) for three 1-minute pulses with 1-minute resting and cooling intervals after which the suspension was centrifuged at 10K rpm for 10 minutes. Since previous purification experiments had shown that the *E. coli* expressed protein was insoluble, the pellet was saved and resuspended in 3 mL lysis buffer. Glycerol was added to a final concentration of 10% and the partially purified extracts were stored at -80°C.

Approximately 0.8 mL of the partially purified protein extract along with 0.2mL SDS sample buffer were loaded into one large well of a discontinuous SDS-polyacrylamide gel (4% stacking, 12% separating) and run at 20 mA until the sample dye was approximately 3/4 down the length of the gel. After trimming the sides of the gel to get rid of the edges, a thin longitudinal slice was made on one side of the gel and quickly stained and destained. The slice was then realigned with the rest of the gel. Over a light box, a thin horizontal slice corresponding to the expressed protein band was cut out and chopped into small 1-cm pieces. The slices were placed longitudinally in the elution funnel of a modified disk gel electrophoresis apparatus (Buchler Instruments, Fort
Lee, NJ) which was used to elute the protein. The elution funnel was plugged at its positive end with 0.5-1 cm of 1% agarose to prevent the gel slices from entering into the collection chamber; electroelution of the protein was overnight at room temperature and 250V. The eluant was collected in a Centricon 10 microconcentrator tube (Amicon, Beverly, MA) and spun at 5000xg and room temperature until the volume was about 200 µL (approximately 60-90 minutes). The tube was then inverted and centrifuged an additional 3 minutes at 5000xg to collect the protein. An aliquot of the protein eluant was run in a 12% discontinuous SDS-polyacrylamide gel to determine the extent of protein purification and recovery.

Anti-NIT3 polyclonal antibodies were raised in New Zealand white rabbits by subcutaneously injecting approximately 150-200 µg of expressed, purified protein. Two booster shots, each of 100 µg protein, were given at 6-week intervals after the initial injection. All injections, bleedings, and animal care were handled by members of Dr. B.S. Zwilling’s lab (Department of Microbiology, Ohio State University).

**Western analysis**

*Neurospora* crude cellular extracts were prepared by grinding on ice 500 mg of mycelia with 0.5 mL of extraction buffer (20 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF) with a mortar and pestle. The extracts were centrifuged at 14K rpm, 4°C for 10 minutes to remove the cellular debris, and the supernatants were transferred to a fresh tube for storage at 4°C.

For Western blots, 12 µL of *Neurospora* whole cell extract with 3 µL of SDS-loading buffer were boiled for 5 minutes, briefly centrifuged and the entire supernatant
loaded onto a 7.5% discontinuous SDS-polyacrylamide gel. The protein gels were
electroblotted onto 2 layers of BA-85 nitrocellulose filter (Schleider and Schuell, Keene,
NH) as described in Sambrook et al. (1989) using the Bio-Rad TransBlot Cell apparatus
and power supply (Model 160/1.6, Bio-Rad Labs, Richmond, CA). Protein transfer was
usually for 2.5 to 3 hours at 55 V and room temperature.

Two different methods were used for detection of the NIT-3 protein, following
in each case the manufacturers’ suggestions. The Immunoselect kit (Bethesda Research
Laboratories, Gaithersburg, MD) employs a biotinylated goat anti-rabbit IgG,
streptavidin-alkaline phosphatase system. After transfer, the filters were blocked
overnight in TBST buffer containing 1% BSA and 0.1% casein at room temperature with
gentle shaking. Preliminary titration experiments had shown that the best results for
protein detection with anti-NIT-3 antibody in Neurospora crude extracts was when it was
diluted 1:1000. Dilution of the secondary antibody, wash times and substrate
concentrations were exactly as prescribed by the manufacturer. After addition of the
substrates, the filters were allowed to undergo the enzymatic reaction for about 5 minutes
in the dark before thoroughly washing several times with deionized water.

The Enhanced Chemiluminescence kit (Amersham Corp., Arlington Heights, IL)
uses a donkey anti-rabbit IgG, horseradish peroxidase-linked secondary antibody for
immunodetection. The blocking procedure involved incubation of the filters in TBST
with 1% BSA and 5% casein for 60 minutes at room temperature with gentle agitation
and then storing in the blocking buffer at 4°C overnight. Because this system is much
more sensitive, the secondary antibody was diluted 1:5000 with TBST and each wash
time was extended to 15 minutes with a large volume of TBST buffer (about 20-25 mL per 2 filters). Dilution of the primary antibody was 1:1000 in TBST, and substrate concentrations and development conditions were as described in the manufacturer’s protocol. After development, the blots were wrapped in Saran plastic wrap and exposed to Kodak XAR film (Rochester, NY) for 3-5 seconds in the dark. With each detection system, specificity was enhanced by preincubating the primary polyclonal antibody with 5-10 μL of *Neurospora* extract from nitrogen-repressed wild-type mycelia per 10 mL hybridization buffer.

**Benzidine staining for heme**

Native gel electrophoresis was performed as described (Clarke 1964). The separating gel consisted of 7.5% acrylamide, 0.375M Tris, pH 8.8 and 1 mM EDTA while the stacking gel was made up of 4% acrylamide, 0.125M Tris, pH 6.8 and 1 mM EDTA. After wrapping the gel in Saran plastic wrap, it was stored overnight at room temperature to insure that all the ammonia persulfate had been destroyed. Sixteen microliters of freshly prepared *Neurospora* cell extracts and 4 μL of sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 0.00125% (w/v) bromphenol blue) were loaded into each well. All electrophoresis experiments were performed at 4°C in Tris-glycine buffer, pH 8.3, and run at 25V until the sample dye eluted out of the gel.

Benzidine staining was carried out essentially as reported elsewhere (Broyles et al. 1979). The 0.2% (w/v) benzidine stain was prepared by dissolving 100 mg of benzidine-HCl (Sigma Chemical Co., St. Louis, MO) in 1M acetic acid to a total volume of 49 mL. Just prior to use, 1 mL of 3% H₂O₂ was added and mixed by inversion. Gels
were stained at room temperature for about 10 minutes or until the lanes not loaded with protein developed into a deep blue color and the bands became discernible. After staining, the gels were rinsed twice with deionized water, which turns the blue stain into brown, and then kept in water overnight. For comparison, a duplicate gel was stained with Coomassie R-250 Brilliant blue dye.

**Enzyme assays**

All enzyme and protein concentration assays were performed in duplicate.

Full nitrate reductase enzyme activity was assayed as described previously (Garrett and Cove 1976). Specific activity was calculated from measurements taken at 10 and 20 minute reaction times.

Reduced methyl viologen-nitrate reductase partial activity was assayed by the procedures of Garrett and Nason (1967) and of Campbell (personal communication). Each assay consisted of 50 μL crude enzyme extract, 50 μL 200 mM KNO₃, 25 μL 10 mM methyl viologen (Sigma Chemical Co., St. Louis, MO) and 0.85 mL 30 mM phosphate buffer, pH 7.4 with 10 mM EDTA. The reaction mixtures in 13x100 mm culture tubes were prewarmed for 5 minutes at 30°C after which freshly prepared sodium dithionite reductant (25 μL 100 mM solution in 0.1 N NaOH) was added to start the redox reaction. Methyl viologen was used as an electron carrier from sodium dithionite in the reduction of nitrate to nitrite. The assay was performed at 5- and 10-minute timepoints, and stopped by vortexing vigorously for about 20 seconds to produce a change in solution color from deep purple to colorless followed by precipitation with 50 μL 1 M ZnOAc. The supernatant was measured for nitrite formation using the diazo
reaction as in the full nitrate reductase assay.

Partial activity of the combined heme-flavin domain was determined by measuring the increase in absorbance at 550 nm in a reaction mixture comprised of 100 μL 0.1 mM FAD, 100 μL 10 mg/mL cytochrome c (Type VI dissolved in phosphate buffer, pH 7.4; Sigma Chemical Co., St. Louis, MO), 100 μL crude enzyme extract and 1.6 mL 30 mM phosphate buffer, pH 7.4 with 10 mM EDTA (Garrett and Nason 1967). After adding 100 μL 2 mM NADPH to start the reaction, the solution was briefly mixed and the rate of cytochrome c reduction was immediately measured at 550 nm and at room temperature.

For partial activity assays of the flavin domain, the reaction composition was identical to that for the heme-flavin assay except that 100 μL of 10 mM potassium ferricyanide replaced cytochrome c, and the rate of NADPH oxidation was measured as a decrease in absorbance at 420 nm (Garrett and Nason 1969). For both heme-flavin and flavin partial assays, it was sometimes necessary to dilute the crude enzyme extract with phosphate buffer to obtain a linear absorbance curve over a 2 minute period. Absorption measurements for the flavin-heme and flavin domain activities were taken on a Gilford spectrophotometer interfaced to a Gilford chart recorder, Model 6051 set at a chart speed of 5 cm/min. Protein concentrations were assayed by the Bradford method (1976) using bovine serum albumin as the protein standard.
CHAPTER III

KINETIC STUDIES OF \textit{nit}-3 mRNA AND PROTEIN

ACCUMULATION AND TURNOVER

Introduction

Although transcription is but one of a number of steps that are involved in the overall process of gene expression and protein synthesis, it is generally the predominant, and sometimes the only, level at which regulation is exerted. Moreover, since transcription itself consists of various steps, control can be achieved at any one of several sites and often occurs as a response to external and/or internal factors. One principal means of regulation is by altering the rate of synthesis and turnover of mRNA. Ubiquitous proteins that are in constant demand generally have mRNAs with much longer half-lives than those proteins whose syntheses are dictated by changing physiological conditions such as the availability of a preferred nutritional source.

Early genetic and biochemical studies established that \textit{Neurospora} nitrate reductase activity is repressible by ammonia and inducible by nitrate (Cove 1979). Numerous \textit{in vivo} studies have indirectly demonstrated that the expression of nitrate reductase is regulated at the transcriptional level (Premakumar and Sorger 1979), requiring both the presence of nitrate and the absence of ammonia (either condition alone
will not suffice), and that the synthesis and turnover of nitrate reductase proceed rapidly under varying metabolic conditions (Premakumar et al. 1978). With the recent cloning of the nitrate reductase gene (Fu and Marzluf 1987a), it has now become possible to directly examine not only the regulation of nitrate reductase expression but also to analyze how rapidly Neurospora can accommodate sudden nutritional changes.

Results

Metabolic Regulation of nit-3 Expression

To directly determine whether the expression of nitrate reductase necessitated both nitrogen derepression and specific induction by nitrate, Northern analysis was carried out. RNA was isolated from wild-type mycelia which were initially grown under repressed conditions with glutamine and then transferred to media of contrasting nitrogen conditions for an additional four hours of growth. Previous studies had found that at least three hours of growth in induction medium was adequate for the expression of nitrate reductase (Subramanian and Sorger 1972a) and has been routinely used to induce expression. As shown in Figure 2, the nit-3 transcript was present in a higher level in cells that were simultaneously derepressed for ammonia and induced with nitrate. The smaller mRNA band represents the constitutively expressed, 1.8 kb β-tubulin transcript which was used as an internal control. In comparison, the size of the nit-3 message is estimated to be about 3.4 kb.

Kinetic Accumulation of nit-3 mRNA and Protein

Nitrate reductase is present only in low cellular concentrations, and is known to be a highly efficient enzyme whose activity rapidly fluctuates in response to sudden
environmental changes. Since de novo enzyme synthesis has already been demonstrated to be required for increases in nitrate reductase activity (Bahns and Garrett 1980), both the synthesis and stability of its mRNA and/or protein could account for these rapid changes in activity. Thus, it was of particular interest to directly determine at the molecular level the efficiency with which Neurospora adapts to abrupt variations in the nitrogen nutritional source available.

Kinetic accumulation of the nit-3 message was studied by Northern blot analysis. Following growth of wild-type Neurospora under nitrogen repressed conditions, the mycelia were transferred to nitrogen derepressed, nitrate induced media. The cells were harvested at varying times and, the amount of nit-3 mRNA was estimated. In preliminary experiments, mRNA was obtained from samples which had been collected at hourly intervals. Yet, the quantity of nit-3 transcript detected after only 60 minutes of induction appeared to already be at the maximum level. Consequently, shorter induction times were used, and the results are presented in Figure 3. After merely 15 minutes of ammonia derepression and simultaneous nitrate induction, nit-3 expression seems to be maximal.

Concurrent with the nit-3 mRNA study, the kinetics of accumulation of nitrate reductase activity were measured (Figure 4). As with the rapid onset of mRNA synthesis, there is also a significant increase in nitrate reductase activity within 15 minutes of induction, reaching a maximal level after about 60 minutes of induction.
Stability of the *nit-3* mRNA and Protein

The stability of the *nit-3* transcript was studied by Northern blot experiments in which mRNA was isolated from wild-type mycelia that had been repressed with glutamine for different times following a uniform period of nitrate induction. Preliminary Northern analyses showed little to no detectable *nit-3* message from mycelial samples which had been repressed for 15- and 30-minute intervals, respectively. Thus, for a more useful analysis, repression times were shortened to 5, 10 and 20 minutes after induction. A rapid turnover of *nit-3* transcript is observed almost immediately upon nitrogen repression, as shown in Figure 5. After only 10 minutes, a substantial amount of the message has disappeared, and by 20 minutes of repression, almost all of the *nit-3* mRNA has been degraded. The half-life of the *nit-3* mRNA was estimated to be approximately 5 minutes (Figure 6).

In parallel to the *nit-3* mRNA turnover study, the cellular level of nitrate reductase activity was determined at various times after ammonia repression (Figure 7). Preliminary enzyme assays on mycelia which had been repressed for longer than 20 minutes revealed that after 60 minutes of ammonia repression, essentially no measurable nitrate reductase activity was present. The calculated half-life for nitrate reductase activity was determined to be about 15 minutes.

Discussion

Three direct lines of evidence provided in these results strongly suggest that the primary regulation of nitrate reductase expression occurs at the transcriptional level. First, no detectable *nit-3* message is present in nitrogen repressed cells, and *nit-3* mRNA
levels increase upon both nitrogen derepression and nitrate induction. Second, the large increase in nitrate reductase activity correlates well with the rapid accumulation of the nit-3 transcript upon induction. Finally, the high turnover rate of the nit-3 mRNA upon nitrogen repression correlates well with a dramatic decrease in enzyme activity under the same condition.

It appears that the cellular level of nitrate reductase is highly dependent upon the kind of nitrogen source available. These results show that the expression of nit-3 requires both nitrogen derepression and nitrate induction. A 50-fold increase in the amount of nit-3 transcript upon induction has been reported (Fu and Marzluf 1987a). Several in vivo studies have also previously examined the effects of other nitrogen conditions on nitrate reductase expression and have revealed that nitrate reductase was synthesized with low efficiency when no nitrogen source was available (nitrogen derepression), but increased significantly when nitrate was added (Subramanian and Sorger 1972b). The addition of ammonia to nitrate medium (nitrogen repression, nitrate induction) was shown to further decrease enzyme activity (Sorger and Davies 1973). More direct evidence obtained from Northern analyses of mRNA isolated from either nitrogen derepressed or nitrogen repressed, nitrate induced mycelia demonstrated that virtually no detectable nit-3 transcript was evident (Fu and Marzluf 1987a), substantiating the earlier indirect studies. Moreover, the lack of nit-3 transcript with mutants of the regulatory genes, nit-2 and nit-4, even under favorable nitrogen conditions, suggested that the regulatory genes have their effect on nit-3 expression at the level of transcription (Fu and Marzluf 1987a). The observed constitutive expression of nit-3 in nit-3 mutants
supports the suggestion that autogenous regulation of nitrate reductase also occurs at the transcriptional level (Fu and Marzluf 1988a).

The accumulation of nitrate reductase activity upon nitrogen derepression and induction by nitrate shows a rapid rise with a leveling of activity between 1 and 2 hours, and agrees with data from previous studies (Subramanian and Sorger 1972a). Although enzyme activity was not measured prior to 15 minutes of induction in this study, earlier findings in Neurospora have demonstrated that the appearance of enzyme activity lags for 15-20 minutes followed by a considerable increase in activity upon induction (Subramanian and Sorger 1972a). This apparent lag in nitrate reductase activity is presumably attributed to a delay in de novo protein synthesis, and has also been observed in other fungi such as the ascomycete, Aspergillus nidulans (Cove 1966) and the basidiomycete, Ustilago maydis (Lewis and Fincham 1970). That is, in these organisms, little nitrate reductase activity was observed immediately after initial transfer to nitrate inducing medium (8 and 30 minute lags for Aspergillus and Ustilago, respectively), that was then followed by a sharp rise in activity, reaching a steady state at about 2 hours.

The rapid increase in nitrate reductase activity under induced conditions appears to be in direct response to the even more rapid accumulation of the nit-3 transcript. Upon nitrogen derepression and nitrate induction, the nit-3 mRNA seems to have reached a steady state level after merely 15 minutes. Thus, these results provide further evidence that the regulation of nit-3 expression occurs primarily at the transcriptional level. However, these experiments cannot totally rule out the possibility that a closely associated step such as mRNA processing may also be involved. One direct approach
to determine more conclusively whether transcriptional regulation is involved in nitrate reductase expression would be to directly measure transcriptional activity using nuclear runoff assays.

As with the correlation between the rapid accumulations of the nit-3 mRNA and protein, the dramatic decrease in nitrate reductase activity seems to also be directly related to turnover of the nit-3 transcript. Direct analysis of nit-3 message stability revealed that rapid turnover occurs upon nitrogen repression. The estimated half-life of the nit-3 mRNA is approximated to be 5 minutes, which agrees well with an earlier estimate (8.5 minutes) determined by indirect methods (Premakumar et al. 1978). The short half-life exhibited by the nit-3 message may account for the ability of Neurospora to readily adjust to differing nutritional challenges. Short half-lives have also been observed with other fungal structural genes whose expression is governed by environmental factors. For example, the half-life of cys-14 mRNA, which codes for sulfate permease II of the Neurospora sulfur circuit, has an estimated half-life of 15 minutes (Ketter et al. 1991), while the half-life for arginase in yeast is 4-5 minutes (Bossinger and Cooper 1977) and in Aspergillus, 2.5 minutes (Cybis and Weglenski 1972).

The half-life of Neurospora nitrate reductase has been determined indirectly to be about 15 minutes. Previous investigations have surmised that this loss of nitrate reductase activity is due to in vivo instability and cessation of protein synthesis and not catalytic inactivation upon repression (Sorger et al. 1974). The rapidity with which nitrate reductase turns over after repression has also been observed in other organisms
such as *Ustilago* (Lewis and Fincham 1970), *Aspergillus* (Cove 1966), *Candida nitratophila* (Cannons and Hipkin 1987) and *Chlamydomonas* (Franco et al. 1987). With one exception, nitrate reductase turnover was attributed to a lack of synthesis and a continual breakdown of the protein. Interestingly, in *Chlamydomonas*, it appears that nitrate reductase is reversibly inactivated and an inactive form of the enzyme exists (Franco et al. 1987). However, despite the fact that the mechanisms of nitrate reductase regulation may vary among different organisms, it is clear that the enzyme is highly controlled in all cases. A more direct approach to determine if the stability of the protein is being affected upon repression would be to directly measure the level of protein present with time by immunological techniques.

It has been suggested that enzymes, such as nitrate reductase, which catalyze the initial or rate-limiting step of an entire metabolic pathway have evolved to turn over rapidly in response to changing nutritional conditions (Goldberg and Dice 1974). In this way, the more adaptable an organism can be, the greater would be its survival during periods of hard nutritional times and in poor environments. The highly regulated expression of the *nit-3* gene allows for speedy responses to induction and to nitrogen repression, such that both *nit-3* mRNA synthesis and turnover occur quickly under the appropriate metabolic conditions. Moreover, since nitrate assimilation is an energetically expensive process, the highly controlled expression of nitrate reductase insures that no unnecessary cellular energy is wasted upon its synthesis should a preferred nitrogen source be readily available. Thus, the rapid synthesis and efficient turnover of *nit-3* mRNA and nitrate reductase represent a strategy which allows the preferential use of a primary nitrogen source.
CHAPTER IV

SEQUENCE AND MOLECULAR ANALYSIS

OF THE nit-3 GENE

Introduction

The nit-3 gene was cloned (Fu and Marzluf 1987a) by screening a Neurospora genomic cosmid library (Vollmer and Yanofsky 1986) using the sib selection method (Akins and Lambowitz 1985), with complementation of a nitrate reductase auxotroph by transformation. From an isolated 40 kb nit-3 positive cosmid, a 3.8 kb EcoRI fragment (subcloned in plasmid pnit-3) was found to be able to transform a nit-3 mutant strain to prototrophy and thus, was assumed to contain the entire nit-3 gene. Restriction fragment length polymorphism analysis further showed very tight linkage of pnit-3 to two genetic markers, cot-1 and 5S gene 4, which are known to be located on either side of the nit-3 locus on linkage group IV (Metzenberg et al. 1984). Therefore, the above results confirmed that the nit-3 gene had been cloned and strongly suggested that it might be contained in its entirety within the 3.8 kb EcoRI fragment of pnit-3 (Fu and Marzluf 1987a). However, in order to fully undertake molecular studies on the structure and function of nitrate reductase, it was imperative to sequence and characterize the nit-3 gene.
Results

Sequence of the nit-3 Gene

The \textit{pnit-3} plasmid was used to make nested deletion subclones in both directions which were sequenced by the dideoxy chain termination method. The "M13 Reverse" primer was employed for all initial sequencing, and from the sequence data, specific primers were designed and used to resolve any sequence gaps between the deletion subclones. Although previous transformation studies had implied that the entire \textit{nit-3} gene was contained in the \textit{pnit-3} plasmid, upon translation of the DNA sequence and subsequent amino acid homology comparison with other nitrate reductase sequences, it was obvious that \textit{pnit-3} lacked a significant portion of the 5'-end of \textit{nit-3}. Southern analysis of the 40 kb \textit{nit-3} positive cosmid, which was digested with several restriction enzymes and probed with a sequencing primer that was specific to the 5'-end of the known sequence, identified a 4.5 kb \textit{BamHI} fragment that hybridized strongly with the probe (data not shown) and was presumed to encompass the entire 5'-end of the \textit{nit-3} gene. This DNA fragment was subcloned into a plasmid that was designated \textit{pnit-3b} and sequenced via nested deletion subclones. Collectively, about 5 kb of DNA was sequenced; sequencing was carried out at least twice on each DNA strand. The sequencing strategy is shown in Figure 8, and the sequence of \textit{nit-3} and its flanking regions is presented in Figure 9.

Direction of Transcription

To determine the direction of transcription, Northern analysis was performed on poly(A)$^+$ mRNA isolated from both nitrogen repressed and nitrogen derepressed, nitrate
induced mycelia. The nit-3 gene was cloned in opposite directions into the pBluescript vector so that single-stranded DNA which was specific for the sense and for the antisense strands of nit-3 could be isolated and used as nonradiolabeled probes. Since the single-stranded probe which hybridized to the nit-3 mRNA would also have pBluescript sequence, the Northern blots were further probed with radiolabeled double-stranded pBluescript vector. A strong signal was detected on the blot probed with only one strand of nit-3 (Figure 10), and this strand was determined to be the sense strand. As shown in Figure 8, the direction of transcription was found to be from left to right on the physical map, and amino acid homology comparisons between the putative nit-3 translated sequence and the nitrate reductase sequences of other fungi and higher plants agree with these results.

Mapping the Ends of the nit-3 Transcript

For all mapping studies, poly(A)+ mRNA was isolated from both nitrogen repressed and nitrogen derepressed, nitrate induced mycelia. Since it has been established that no nit-3 message is present under nitrogen repressed conditions (Figure 2), experiments with poly(A)+ mRNA from nitrogen repressed cells were useful as a negative control to detect any nonspecific reactions that may occur.

The 5'-end of the nit-3 transcript was determined by performing both standard S1 nuclease mapping and primer extension analyses as described in Chapter II. S1 nuclease mapping experiments utilized an 870 bp BamHI-XbaI fragment which was uniquely end-labeled at the BamHI site for hybridization to the poly(A)+ mRNA. As can be seen in Figure 11, a single fragment of about 570 bp was protected from S1-nuclease digestion
and corresponds to a site approximately 100 bp upstream from a putative ATG translational start codon. To define the transcriptional start site more precisely, primer extension analysis was carried out using a 17-mer of the sequence 5'-GGTCGTTGTCAGTTCA-3' which was positioned 155 bases downstream from the putative translational start site. The results are given in Figure 12. Three transcriptional start sites, each beginning with a purine base, are located within a 20 bp region, with the first start site 110 bp from the putative translational start site (Figure 9). These results agree well with the data from S1 nuclease mapping, in that the close proximity of the three transcriptional start sites to each other accounts for the presence of only one apparent S1 nuclease-protected fragment.

To determine the 3' end of the nit-3 transcript, mapping by S1 nuclease protection was carried out, using a uniquely end-labeled 1 kb BglII-KpnI fragment as the probe. The results are presented in Figure 11, and show a protected fragment of approximately 610 bp which is strongly visible only in the nitrogen derepressed, nitrate induced mRNA lane. The 3'-end of nit-3 mRNA appears to about 50-60 bp from a putative translational stop site.

Isolation and Analysis of cDNA Clones

A λgt10 cDNA library made from Neurospora mRNA isolated from nitrogen derepressed, nitrate induced mycelia was screened with the nit-3 gene, which identified a number of positive plaques. After three rounds of plaque purification, purified phage DNA was isolated from 12 plaques and cloned into the pBluescript vector. From this screen, the largest cDNA fragment was determined to be 1.6 kb by Southern analysis
(data not shown). Upon sequencing, this clone was found to represent only a partial $nit$-$3$ cDNA whose sequence corresponded to the 3'-half of the genomic $nit$-$3$ sequence with the exception of a 61 bp intron that interrupted the open reading frame near the 3' end (Figure 9 and 13A). In addition, this cDNA clone also possessed a poly(A) tract comprised of 15 deoxyladenylate residues (Figure 13B) that were 48 bases downstream from the putative TGA translational stop codon (Figure 9). The position of the 3'-end of the transcript agreed well with the results from S1 nuclease mapping experiments. Although several attempts were made to isolate a larger cDNA clone or a cDNA clone that encompassed most or all of the 5'-end, no such clones were found. Using the extensive amino acid homology between the $nit$-$3$ translated sequence and nitrate reductases of other organisms, (especially Aspergillus), as well as a combination of reading frame data and splice site consensus sequences for Neurospora, it was concluded that no other introns were present in the $nit$-$3$ gene. A major concern was that an intron might be present at the 5'-end of $nit$-$3$ since the extreme N-terminus (about the first 100 residues) seemed to lack significant homology with other nitrate reductases. However, results from S1 nuclease mapping experiments of the 5'-end showed a single protected fragment that corresponded to 570 bp (or 190 amino acids) of the coding region and confirmed the other data that indicated no introns existed within this region of the gene (Figure 11).

Discussion

The nucleotide sequence, transcript size and direction of transcription have been determined for the $nit$-$3$ gene. S1 nuclease mapping, primer extension and cDNA
sequence analyses revealed that the \textit{nit-3} transcript is 3107 nucleotides in length, which concurs well with the size estimated by Northern analysis. The \textit{nit-3} gene encodes a protein of 982 amino acid residues with a calculated pI of 5.68 and a molecular weight of 108 kDa. This calculated molecular weight closely agrees with a previously published value of 110 kDa (Bahns and Garrett 1980). Comparison of the translated \textit{nit-3} sequence to a partial \textit{Neurospora} nitrate reductase sequence (Lê and Lederer 1983) determined from direct protein sequencing showed them to be very similar; the slight discrepancies observed were probably due to species-dependent differences or experimental error. Thus, it was ascertained that the previously cloned \textit{nit-3} gene does, indeed, code for nitrate reductase in \textit{Neurospora crassa}.

Perusal of the \textit{nit-3} nucleotide sequence has revealed some interesting, significant features about the gene. Three potential transcriptional start sites occur at closely spaced positions of +1, +10 and +22 with the first start site immediately preceded by a long, 29 bp pyrimidine tract which is comprised primarily of thymidine residues (Figure 9). Long pyrimidine stretches have also been observed in a variety of \textit{Neurospora} genes (Fu and Marzluf 1990a; Lee \textit{et al.} 1990a; Young \textit{et al.} 1990; Ketter \textit{et al.} 1991) as well as other fungal genes (Lue \textit{et al.} 1989). Although the significance of this motif is as yet unknown in \textit{Neurospora}, it has been postulated with the \textit{nit-2} gene to be involved in mRNA translation or stability (Fu and Marzluf 1990a). This is not an unreasonable assumption since oligopyrimidine tracts up to 29 residues have been demonstrated in mammalian ribosomal protein genes to be important in translational regulation of the mRNA (Levy \textit{et al.} 1991). On the other hand, lengthy thymidine-rich regions have also
been implicated to act as upstream activation sequences that are involved in transcriptional activation via protein exclusion for several genes (Struhl 1985). However, this is not always the case as was shown by Lue et al. (1989) in which a thymidine-rich region upstream of the *Saccharomyces cerevisiae* gene, DED1, activated transcription *in vitro* not by protein exclusion as suggested for some other yeast genes but possibly through a protein-DNA interaction with one member of a family of thymidine-rich DNA-binding transcription factors that are specific for different thymidine-rich motifs.

In addition to the long oligopyrimidine tract, a putative CAAT motif occurs at position -106, and a short sequence, TACC, is repeated eight times in tandem from -63 to -97 just prior to the initiation codon (Figure 9). The significance of this repetitive sequence is unknown, although it might function in translational regulation. Even though a potential TATA box element, TATACATA, is present at position -90 bp (Figure 9), *in vitro* DNA-binding assays with *Neurospora* TFIID protein have demonstrated that TFIID does not bind to the nit-3 5'-promoter fragments (G. Jarai, personal communication), suggesting the absence of any genuine TFIID binding site in the nit-3 promoter region. In contrast, DNA footprinting experiments with the NIT-2 protein have demonstrated that binding requires a short hexameric sequence, TATCTA, which is localized in the -70 bp region of the nit-3 gene (Figure 9; Fu and Marzluf 1990b). Moreover, this sequence has been shown to be highly homologous with sequences located in the common 5'-nontranslated region of the divergently transcribed *Aspergillus* nitrate and nitrite reductase genes. The context of the putative ATG initiation site agrees well (56%; 9 of 16 bases) with the consensus sequence for *Neurospora* (Roberts et al. 1988),
and no other ATG codon occurs upstream of the initiation site shown in Figure 9.

The nit-3 transcript ends 48 nucleotides after a putative TGA termination codon, and a possible polyadenylation signal, AATGAA, is found 23 bp upstream of the polyadenylation site (Figure 9). Although the presence and significance of potential polyadenylation signals have not been well established in Neurospora, hexameric sequences like this one have been shown in Xenopus oocytes to yield a reduced level of 3'-mRNA processing (Wickens and Stephenson 1984). Immediately following the nit-3 poly(A) addition site, a block of guanine and thymidine residues occur and have the consensus sequence for the "G/T cluster" (Figure 9). These motifs occur within 30 bp downstream from the polyadenylation site in a number of eucaryotic mRNAs and are known to be important for 3' processing of poly(A)+ mRNA (Birnstiel et al. 1986). As in the 5'-nontranslated region, a short TGG sequence is tandemly repeated 11 times in the 3'-nontranscribed region of the nit-3 gene (Figure 9). Its significance, however, is unknown.

The single nit-3 intron is located near the 3'-end of the gene, and its sequence agrees completely at the 5'- and 3'-splice sites and at the internal branch site to the consensus sequence for Neurospora introns (Orbach et al. 1986). On the other hand, the related ascomycete, Aspergillus nidulans, has 6 introns in its nitrate reductase gene, niaD (Johnstone et al. 1990). Comparison with the nit-3 intron has revealed that the locations of the nit-3 intron and intron VI of niaD occur at the same site and in both cases, interrupt the same histidine codon, CAC, between the first and second base. This conservation of location between the two introns, thus, raises the possibility that the
intron sequences may themselves be related. In fact, alignment of the two intervening sequences shows that the 5'-splice sites are identical as are 16 of 21 bases (76%) just upstream of the 3'-splice sites for the nit-3 and niaD genes (Figure 14). The remaining sequences of the two intron completely differ from each other. Interestingly, whereas the nit-3 gene contains only one intron, the nitrate reductase genes in Aspergillus and higher plants have six and two to three intervening sequences, respectively (Caboche and Rouzé 1990). Furthermore, unlike intron VI of niaD, sequence comparison of the nit-3 intron with introns I, II, III, IV and V reveals less than 50% homology (Figure 14) and suggests a common origin for the nit-3 intron and intron VI of Aspergillus. Conceivably, evolutionary divergence between the nitrate reductase genes of these two related filamentous fungi resulted in either Neurospora losing or Aspergillus gaining five extra intervening sequences. The positions of the introns in higher plants occur at unique sites and do not correlate with those found in nit-3 or niaD.

*Neurospora* nitrate reductase is a biochemically well-characterized homodimeric protein with each subunit consisting of three discrete binding domains for the cofactors, molybdenum, heme and FAD, which are essential for enzymatic activity. In order to identify the individual amino acids that compose the functional domains of the enzyme, the deduced nit-3 translated sequence was compared to the nitrate reductase sequences of *Aspergillus nidulans* (Johnstone et al. 1990), *Arabidopsis thaliana* (Crawford et al. 1989), *Nicotiana sylvestris* (tobacco; Calza et al. 1987; Galangau et al. 1989), *Lycopersicon esculentum* (tomato; Daniel-Vedele et al. 1989), *Zea mays* (corn; Gowri and Campbell 1989), barley (Cheng et al. 1986), *Curcurbita maxima* (squash; Hyde et
al. 1991) and *Chlamydomonas reinhardtii* (Fernandez *et al.* 1989). Significant homologies, particularly within the cofactor-binding domains were identified (Figure 15). Not surprisingly, *Aspergillus* nitrate reductase showed the strongest homology with the analogous *Neurospora* protein, with approximately 55% identity primarily within the cofactor-binding domains of the two fungal enzymes. A common evolutionary origin is reflected by the extensive homology observed between the *Neurospora* nitrate reductase sequence and those of algae, higher plants and *Aspergillus*. The differences found between fungal and higher plant nitrate reductase sequences are apparently a result of evolutionary divergence.

Comparison with functionally similar but physiologically unrelated redox enzymes that bind molybdenum, heme or FAD have revealed significant sequence similarities to corresponding regions in the *nit-3* protein and suggest probable positions of these functional domains in nitrate reductase. Catalysis via electron transfer is initiated in the FAD domain and proceeds through the heme and then the molybdenum domain. Alignment of the nitrate reductase sequences and the unrelated redox proteins: chicken and rat liver sulfite oxidase (Crawford *et al.* 1988; Neame and Barber 1990), human liver cytochrome b₅ (Yoo and Steggles 1988) and human erythrocyte NADH-cytochrome b₅ reductase (Yubisui *et al.* 1986), have revealed that the cofactor binding domains are linearly organized opposite to that of catalysis, beginning with the molybdenum domain in the N-terminal region followed by the heme domain in the middle and the FAD domain in the carboxyl end. Moreover, combined with previous results from limited proteolysis studies (Kubo *et al.* 1988), these sequence homologies define putative
boundaries for the molybdenum, heme and FAD binding domains, respectively, in the primary sequence of *Neurospora* nitrate reductase. Hence, the heme-binding domain for the *Neurospora* nitrate reductase is tentatively assigned to encompass a 70 amino acid region bordered by Thr 621 and Leu 691 while the FAD binding domain begins at Leu 718 and extends to the end of the protein (Figures 15 and 16). The boundaries for the molybdenum domain are estimated to be from Ile 139 to Val 550, giving a domain of approximately 400 amino acids in size. Limited proteolysis experiments with spinach nitrate reductase have, furthermore, indicated that protease sensitive hinge regions appear to separate the functional domains into discrete redox centers (Kubo *et al.* 1988). In contrast to the cofactor binding domains in which the active site amino acids are very well conserved, the hinge regions appear to have little sequence agreement and even vary greatly in length among the nitrate reductase sequences (Kinghorn and Campbell 1989 and Figure 15). However, despite the lack of extensive homology within these regions, proteolysis studies have demonstrated that they are important for catalytic activity. In an evolutionary sense, it has been postulated that genetic sequences for proteins with single redox centers were shuffled and fused together to form polyfunctional proteins such as nitrate reductase (Guiard and Lederer 1979), thereby accounting for the extensive sequence similarities with functionally unrelated redox proteins.

The significant amino acid homologies exhibited between the deduced *nit-3* protein, the molybdenum-containing sulfite oxidase, the heme-binding mammalian cytochrome b$_5$, and the FAD-associated cytochrome b$_5$ reductase have helped to identify specific amino acids that may actively play a role in catalysis. Comparison of a number
of cytochrome b₅ sequences has previously revealed 15 invariant amino acid residues that are present in all of the cytochrome b₅ proteins examined and which have been proposed to be important in structure or function (Guiard and Lederer 1979). Of these, *Neurospora* nitrate reductase agrees with 14, including two histidine residues, His 652 and His 675 (Figure 15), that are hypothesized to function in directly binding the heme moiety (Guiard and Lederer 1979).

Analysis of sequence similarities between the predicted nit-3 protein and cytochrome b₅ reductase have also identified potentially important amino acid residues. The flavin domain has been postulated to possess separate binding sites for FAD and NADPH; a cysteine residue, Cys 956, which is fully conserved in all of the nitrate reductases and in cytochrome b₅ reductase is believed to comprise part of the NADPH binding site (Figure 15; Hackett *et al.* 1986; Hyde *et al.* 1989). Moreover, a lysine residue, Lys 796, is also conserved throughout all of the analyzed sequences (Figure 15), and its ε-amino group is thought to also be important in NADPH binding as was observed in an unrelated NADPH flavoprotein (Hackett *et al.* 1988). In all, a total of 15 invariant amino acids, including the aforementioned Cys 956 and Lys 796 residues, have been discerned from the comparison of a number of different flavoenzymes with plant and fungal nitrate reductase primary sequences (Hyde *et al.* 1991).

The molybdenum-binding domain is the largest of the three domains and it is the least examined primarily because there are very few known molybdoproteins that show any homology to it. Comparison of partial chicken and rat sulfite oxidase sequences with various nitrate reductase sequences by Barber and Neame (1990) revealed a single
conserved cysteine residue in all of the homologous molybdenum-binding enzymes analyzed. For *Neurospora* nitrate reductase, this cysteine residue corresponds to Cys 240 (Figure 15). In this analysis, however, another conserved cysteine residue was found at position 295 (Figure 15) for all of the nitrate reductase sequences and chicken liver sulfite oxidase (Figure 15). Immunological studies with squash nitrate reductase protein have revealed that a possible disulfide bond exists between the molybdenum domains of the two subunits (Hyde *et al.* 1989). One of the highly conserved cysteine residues observed in this domain may be involved in intermolecular disulfide bond formation and serve a structural role in stabilizing the dimers rather than a catalytic one. In contrast, the conserved cysteine residue (corresponding to Cys 240 of the *Neurospora* nitrate reductase sequence in Figure 15) in sulfite oxidase has been postulated to bind the molybdenum cofactor through its sulfhydryl group (Barber and Neame 1990). Thus, it may be that one conserved cysteine residue of nitrate reductase is involved in dimer formation while the other interacts directly with the molybdenum cofactor.

One means of predicting protein structure is to look at the nature of neighboring amino acids in the primary sequence. Possible secondary structural motifs were determined for the deduced nit-3 sequence, and revealed some stretches of \(\alpha\)-helices and \(\beta\)-sheet structures (Figure 17) with a number of turns (not all are shown in Figure 17). However, predictions of secondary structure based solely on the primary sequence carry a significant amount of uncertainty and can only be used as a very preliminary guess as to the structures within a protein. A more accurate way to predict tertiary structure of a protein is by comparison with a homologous protein of known crystal structure,
obviously the more similar the primary sequences of two proteins are to each other, the more likely their protein conformations will also agree. In that respect, the crystal structures of two proteins, bovine cytochrome b$_5$ (Mathews et al. 1972) and spinach ferredoxin reductase (Karplus et al. 1990), which have homology to the heme- and FAD-binding domains, respectively, of nitrate reductase have been valuable in making preliminary predictions on the conformation of the protein, or at least two of its three domains, based solely on conserved regions in the primary sequence. For instance, two invariant amino acids of nitrate reductase, Arg 777 and Tyr 779, have been theorized to directly bind to the FAD cofactor (Hyde et al. 1992) as has been shown directly with the crystal structure of ferredoxin reductase (Karplus et al. 1991). The 3-D structure for the heme-binding domain of bovine cytochrome b$_5$ (Mathews et al. 1972) has similarly been useful in predicting conformation of the nitrate reductase heme domain. An antiparallel $\beta$-barrel structure that binds the FAD cofactor in ferredoxin reductase could not, however, be precisely determined for nitrate reductase although some amino acids for this secondary structural motif do occur in squash and Neurospora nitrate reductases (Hyde et al. 1991). Thus, despite the lack of an x-ray crystal structure for Neurospora nitrate reductase, a significant amount of information has been obtained from a combination of structural modeling with proteins of known conformation, secondary structure predictions and analysis of amino acid sequences in conserved regions of a number of nitrate reductases. This information will permit for future studies a rational identification of those amino acid residues that may play critical roles in enzyme structure and function.
CHAPTER V
ANALYSIS OF CONVENTIONAL nit-3 MUTANTS AND
GENERATION OF A NEW nit-3 MUTANT STRAIN BY THE RIP PROCESS

Introduction

The nitrate reductase enzyme of *Neurospora crassa* provides an attractive system in which to undertake protein structure-function studies for several reasons. The straightforward selection of nitrate-requiring mutants on chlorate medium, which is toxic for wild-type cells, has permitted the isolation of a number of nit-3 mutants. The relatively recent advances in *Neurospora* transformation assays have made it simple and efficient to introduce mutated genes into the genome. Moreover, because homologous recombination is an extremely rare event in *Neurospora* and transforming DNA is usually ectopically integrated into the genome, transformation assays allow a rapid and easy means to assess the function of manipulated genes. Finally, the fact that cofactor-binding domains can be individually assayed for activity provides a valuable method in which to localize mutations to specific regions of the protein as well as to evaluate their effects on protein structure and function.

One approach to studying the amino acid structure-function relationships of *Neurospora* nitrate reductase is by molecular analysis of previously isolated conventional
mutants of \textit{nit}-3. In earlier studies, Garrett and Tomsett (1979; 1981) generated numerous \textit{nit}-3 mutants via UV radiation and biochemically characterized them to approximately localize their mutation sites in the protein. Results of partial enzyme activity assays of two of these mutants, V1M16 and V1M4, as well as another conventional \textit{nit}-3 mutant strain, 14789\textit{A}, which is routinely used as the host for \textit{nit}-3 transformation assays, suggested that each harbored different kinds of mutations at the \textit{nit}-3 locus. Curiously, mutations in strains V1M16 and 14789\textit{A} were, furthermore, found to alter the regulation of nitrate reductase synthesis, presumably by affecting the structural integrity of the protein. Thus, it was of interest to directly identify the mutation in each of these mutant \textit{nit}-3 alleles and to determine whether these changes, indeed, affected the regulation of nitrate reductase expression. The \textit{nit}-3 mutant alleles were analyzed by employing the polymerase chain reaction method. Furthermore, polyclonal antibodies specific for the nitrate reductase protein were used to study the altered regulation of nitrate reductase synthesis in these mutants.

In contrast to biochemical and genetic analysis of mutants, a more straightforward approach to examine structure-function relationships in proteins is via \textit{in vitro} mutagenesis which permits specific changes in the structural gene that are then assessed \textit{in vivo}. Since the success of this method is highly dependent upon a suitable host strain that does not display homologous recombination with the mutated transforming DNA, the \textit{Neurospora} transformation system is generally ideal. However, as mentioned in Chapter IV, previous transformation experiments had suggested that the \textit{nit}-3 gene may undergo a higher frequency of homologous recombination when introduced into the \textit{nit}-3 host
strain (14789A) than found with most other Neurospora genes. The results of structure-function studies, which rely on an ectopic integration of the mutated gene into the genome, would be difficult to interpret if complicated by recombination of the mutated transforming gene with the mutant resident gene. Thus, to carry out structure-function studies on nitrate reductase, it was essential to initially construct a nit-3 mutant host strain which had the entire nit-3 locus either deleted or completely disrupted such that homologous recombination could not conceivably occur.

Results

Molecular Analysis of Three nit-3 Mutant Alleles

To directly determine the kinds of mutation that occur in each of the three nit-3 mutant alleles, 14789A, V1M16 and V1M4, the genes were cloned by the polymerase chain reaction method (PCR) and sequenced using the standard dideoxy termination procedure. Partial enzyme activity assays had tentatively localized all of the mutations to be present within the protein coding region of nit-3. That is, the mutations for V1M16 and 14789A were estimated to be in the 3’-region of the gene while the mutation for V1M4 was believed to be in the 5’-end (Tomsett and Garrett 1981). The relatively large size (about 3 kb) of this gene made it difficult to amplify in its entirety by PCR. Consequently, the nit-3 coding region was amplified into approximately 1.1 kb partially overlapping fragments using three sets of primers. The amplified fragments were then subcloned into the pBluescript vector and sequenced. Since Taq polymerase misincorporates nucleotides at a relatively high rate (2 x 10⁻⁴; Saiki et al. 1988), at least three independently isolated colonies from duplicate PCR reactions were sequenced for
each mutant allele. In Figure 18A-C, the precise mutation in each of the strains is presented, and their relative locations in the nit-3 gene are shown in Figure 19. These results agree very well with biochemical data in that the previously observed lack of partial activity for the FAD domain for mutant alleles 14789A and V1M16 correlates to two different single base deletions found in the 3'-end of the gene. Likewise, the absence of any partial activities as earlier shown for the mutant allele V1M4 agrees with the molecular results in that a leucine codon (TTA) that occurs early in the deduced nit-3 protein has been mutated to a nonsense codon (TAA), thereby producing the observed null phenotype.

Expression and Purification of a Partial Nitrate Reductase Protein

The expression of relatively large eucaryotic proteins such as the Neurospora nitrate reductase monomer, which consists of 982 amino acid residues, is usually difficult to achieve in E. coli. Therefore, it appeared necessary to express only a part of the enzyme for antibody production. In addition, it was desirable for future structure-function studies to express one domain in its entirety. Hence, I decided to attempt to express the entire heme-binding domain due to its small size and short hydrophilic regions.

The cloning of the nit-3 fragment, which contains the sequence for the entire heme domain plus some flanking sequences, into the BamHI site of the pET3c expression vector was carried out as described in the Experimental Procedures. The BamHI site of the expression vector was used because it was believed that the inclusion of some E. coli protein sequence may enhance and stabilize the expressed protein. The ensuing plasmid,
designated \textit{pETNIT3ES}, was designed to express an \textit{E. coli} fusion protein which consisted of 204 amino acids of \textit{Neurospora} nitrate reductase that was bordered by 12 and 19 amino acids encoded by the \textit{E. coli} expression vector at the amino and carboxyl ends, respectively, (Figure 20) to give a protein of approximately 28 kDa in size. Sequencing of the fusion junction between the \textit{nit}-3 fragment and the expression vector ensured that the cloned fragment was in the correct reading frame for expression. The \textit{pETNIT3ES} plasmid was transformed into the \textit{E. coli} strain \textit{BL21(DE3)pLysS} and optimal conditions for expression were determined as described in Experimental Procedures. Figure 21A shows an abundantly expressed 28 kDa protein in IPTG-induced \textit{E. coli} cells at the position expected for the NIT3-\textit{E. coli} fusion protein. This protein was purified by electroelution from SDS-polyacrylamide gels and used for polyclonal antibody production (Figure 21B).

The specificity of the anti-NIT3 antibody was initially tested with \textit{E. coli} cellular extracts which contained the expressed partial NIT-3 protein. Induced and uninduced \textit{E. coli} cellular extracts were run in duplicate 12.5\% discontinuous SDS-polyacrylamide gels. One gel was stained for protein with Coomassie Brilliant blue whereas the other gel was electroblotted onto a nitrocellulose filter and probed with the anti-NIT3 antibody. Figure 21C shows that the polyclonal antibody specifically recognizes the expressed partial NIT3 protein in the induced \textit{E. coli} extract.
Western Analysis of the Nitrate Reductase Protein in Wild-type and Mutant *nit-3* *Neurospora* Strains

Crude cellular extracts of *Neurospora* strains 74A, 14789A, V1M16 and V1M4 were prepared from mycelia grown in both nitrogen repressed and in nitrogen derepressed, nitrate induced medium and electrophoresed in duplicate 7.5% SDS-polyacrylamide gels. The 7.5% polyacrylamide concentration not only allowed for the resolution of the fairly large full length nitrate reductase subunit but also permitted the detection of the smallest possible polypeptide (about 66 kDa) that could be detected with the anti-NIT3 antibody (specific for the heme domain). One gel was stained for protein to insure that equal amounts were present in each lane, and the other gel was analyzed via a Western blot. The wild-type *Neurospora* nitrate reductase subunit size is estimated to be 108 kDa as predicted from the *nit-3* nucleotide sequence. As shown in Figure 22, the anti-NIT3 antibody specifically detects a protein of this size but only in the nitrogen derepressed, nitrate induced wild-type *Neurospora* cellular extract. As expected, no protein was detected by the antibody in the nitrogen repressed wild-type extract. These results agree with previous studies which showed that the steady-state level of the nitrate reductase protein is highly controlled by the prevailing nitrogen source (see Chapter III). Moreover, Western analyses of the mutant strains 14789A and V1M16 revealed mutant nitrate reductase proteins truncated to approximately 84 kDa and 100 kDa, respectively, which correlate very well with the position of the mutation found in each of these strains by PCR and sequencing studies. Interestingly, these mutations, a single base deletion in each strain, also somehow affect the regulation of nitrate reductase synthesis such that
the proteins are expressed constitutively (Figure 22). Not surprisingly, the anti-NIT3 antibody did not detect any V1M4 protein (Figure 22) as anticipated from molecular analysis that identified a stop codon very early in the protein sequence (Figure 19).

**Transformation of Host Strain 14789A with Partially Deleted nit-3 Genes**

To address the possibility that the *nit-3* gene undergoes homologous recombination at a frequency unusually high for *Neurospora* genes, nested deletion subclones of *nit-3* were made in both directions and transformed into the strain 14789A. Deletion subclones of special interest were plasmids, pN3Δ3UT, pN3Δ620 and pN3Δ287, which, respectively, lacked the entire 3'-nontranslated region, the coding region for the entire molybdenum-binding domain and a region which overlapped the mutation site for the resident *nit-3* locus. Transformants were selected for their ability to grow in nitrate medium, and as shown in Figure 23, all of the partially deleted *nit-3* genes, except for one, were able to transform the host strain to prototrophy. The number of transformants decreased dramatically from 360-450 transformants per pmole DNA obtained with the complete genes, to less than 25 per pmole DNA for subclones that contained any deletion into the *nit-3* coding region. As anticipated, pN3Δ287 was the only subclone which could not restore the ability of strain 14789A to grow in nitrate. Thus, these results not only suggested that *nit-3* was able to target to its resident locus in the mutant host strain 14789A, but also supported my previous finding that only one mutation site existed in strain 14789A, and that it occurred in the FAD domain. However, the possibility that some other event, such as complementation, may also be involved could not completely be ruled out based solely on the data obtained in this study.
Phenotypic Characterization of the Deletion Transformants

In order to verify that the acquired nitrate-utilizing ability of the deletion transformants was due to the synthesis of an active nitrate reductase, a representative group of the deletion transformants were assayed for nitrate reductase activity. In Figure 24, a clearly measurable nitrate reductase activity is present for each transformant assayed regardless of the size of the deletion. Since the purpose of this study was to simply show that the positive transformants possess a functional nitrate reductase, homocaryons were not isolated. Therefore, this might account for the lower levels of enzyme activity observed among the deleted transformants relative to wild-type strain 74A. Nonetheless, nitrate reductase activity is present in these transformants which suggests that homologous recombination has given rise to a wild-type nit-3 gene.

Southern and Western Analyses of the Deletion Transformants

Southern analysis was used as one approach to assess whether homologous recombination was actually taking place with the nit-3 gene. Using two independent colonies of each of the previously assayed deletion transformants, Neurospora genomic DNA was isolated and digested with EcoRI, which is known to give a fragment of 3.8 kb when probed with the 3'-half of the nit-3 gene. The results show a single nit-3 specific band, which is the identical in size to the wild-type DNA fragment, for most of the transformants (Figure 25), in agreement with the possibility that homologous recombination at nit-3 occurred in the host strain 14789A. However, since the nit-3 mutant allele 14789A also displays the same 3.8 kb EcoRI fragment and extra nit-3 bands were observed in some of the transformants, it was not conclusive that only homologous
recombination and not complementation had taken place.

A more definitive way to show that homologous recombination has occurred in *Neurospora* cells transformed with partial nit-3 subclones was to directly determine the size of the enzyme protein by Western analysis. Only homologous recombination could allow formation of a full-size nitrate reductase subunit. Crude cellular extracts were prepared from nitrate-induced mycelia of the wild-type strain, 74A, the host strain, 14789A and the representative transformants, *pN3RVK*, *pN3PΔ33*, *pN3RΔ205* and *pN3BΔ317*. The extracts were loaded onto duplicate 7.5% SDS-polyacrylamide gels, one of which was stained with Coomassie Brilliant Blue dye to show that uniform amounts of protein were loaded in each lane (data not shown). The other gel was electroblotted and probed with the polyclonal anti-NIT3 antibody for detection of the NIT3 protein. A large protein of approximately 95 kDa was found to cross-react with the streptavidin-alkaline phosphatase-linked secondary antibody in a control experiment (data not shown). This cross-reaction has also been observed with other *Neurospora* crude extracts grown under completely different conditions and is believed to be streptavidin-specific (Jarai and Marzluf 1991a). The presence of this 95 kDa protein, however, proved to be useful as an internal control to show that equal quantities of protein had been transferred to the filter for each lane. Figure 26 shows the Western analysis of the transformants and reveals that for all of the deletion transformants examined, a protein of the same size as the wild-type nitrate reductase subunit is present. Because heterocaryotic cells were used, the endogenous mutant nitrate reductase monomer was also detected. Taken in combination with the previously described results, these data clearly demonstrate that the
nit-3 gene undergoes significant homologous recombination when transformed into the nit-3 mutant host strain 14789A to produce a complete and functional nitrate reductase protein.

Generation of a nit-3 Mutant Host Strain by the RIP Phenomenon

To construct a new nit-3 mutant strain in which the resident nit-3 locus was so badly damaged that targeting could not occur, the recently discovered phenomenon called "RIP", or repeat-induced point mutation (Selker et al. 1987), was exploited. A nit-3 plasmid containing a 3.8 kb Xbal-KpnI fragment which encompassed the entire nit-3 coding region plus 3' - and 5' - flanking regions (Figure 8) was cotransformed with the hygromycin-resistant gene into wild-type strain 74A spheroplasts, resulting in a large number of hygromycin-resistant transformants. The hygromycin-resistant gene was used as a selectable marker since it has no resident locus in wild-type Neurospora and thus, remains stable through crosses. To enhance the mutation frequency in the ensuing cross with wild-type, homocaryons were isolated from several of the heterocaryotic transformants. The isolation of homocaryons prior to crossing has previously been shown to be an efficient method for increasing the frequency of RIP mutants (Jarai and Marzluf 1991b). Thirty hygromycin-resistant homocaryotic colonies were crossed with wild-type. Ascospores were initially germinated in medium containing hygromycin B and nitrate; however, among those that appeared to be auxotrophic, none grew upon transfer to minimal medium supplemented with hygromycin, suggesting that the hygromycin-resistant gene did not cosegregate with the nit-3 gene during the cross. Thus, random ascospores were subsequently plated in minimal medium; from a total of 144 colonies
that were tested, 12 were displayed the nit-3 mutant phenotype; i.e., they grew well in chlorate but not in nitrate media. Since it has been shown that additional rounds of crossing result in even more mutagenesis by RIP, 6 of the 12 progeny from the first cross (F1) that were found to have multiple copies of the nit-3 gene were crossed again with wild-type. Progeny from this second cross (F2) were examined for their ability to grow on nitrate or in the presence of chlorate. Tables 1 and 2 give the results of testcrosses between the established nit-3 mutant host strain 14789A and the F1 and F2 mutant progeny. Progeny from these testcrosses were scored for their ability to utilize nitrate, and all were unable to grow in nitrate. This strongly indicated that new mutants of the nit-3 gene had been generated. When they were crossed with the wild-type strain (74A), about half of the progeny tested were nitrate non-utilizing, as expected (Tables 1 & 2).

Analysis of the nit-3 RIP Mutants by Southern Blot Experiments

Southern analysis was performed on selected F1 and F2 RIP progeny. Their genomic DNA was digested with either PstI or EcoRI, each of which has a single restriction site within the nit-3 gene, and probed with a 1.5 kb HindIII-BglII fragment of the nit-3 coding region. A DNA sample from wild-type 74A was included as a marker for size and copy number. Most of the progeny revealed a change from the wild-type PstI restriction pattern, indicating that the original PstI site had been lost due to mutation (Figure 27). However, the resulting PstI fragment was very large and it was difficult to determine the number of copies that had integrated into the genome. On the other hand, digestion with EcoRI provided a more decisive pattern, and as shown in Figure 28,
all of the F1 progeny examined seemed to have more than one copy of the \textit{nit-3} gene. Further analysis of selected F2 progeny revealed a radical difference in restriction pattern from that of wild-type. In addition, for several of the F2 mutant progeny, it appeared that the original \textit{EcoRI} site had been mutated and a new \textit{EcoRI} site had been created elsewhere in the \textit{nit-3} gene. Of particular interest were two F2 progeny, RIP22A and RIP24A, which seemed to have a single badly damaged copy of the \textit{nit-3} gene as surmised by a decrease in hybridization with the wild-type \textit{nit-3} probe although lower stringency conditions were used in the Southern blot experiments. Thus, these two F2 mutant progeny along with their F1 parent were further analyzed.

Determination of the Extent of Damage by RIP in the New \textit{nit}-3 Mutant Strains

The extent of mutational damage by the RIP process on enzyme function was assessed by nitrate reductase and by partial enzyme activity assays. Mutant RIP alleles RIP17A, RIP22A and RIP9A, along with the wild-type strain 74A and the conventional \textit{nit-3} mutant strain 14789A, were assayed for nitrate reductase activity and for partial activities in the flavin and molybdenum-heme domains as described in Experimental Procedures. Table 3 shows that neither nitrate reductase nor partial enzyme activities were detectable in any of the RIP mutant strains, strongly suggesting that the RIP-induced mutagenesis was extensive throughout the \textit{nit-3} gene.

To further ascertain the extent of damage caused by the RIP process, mutant allele RIP22A was cloned by PCR and sequenced. The first 1000 bases and the last 1000 bases were amplified by PCR and cloned into the pBluescript vector for sequencing. Several attempts to amplify and clone the middle 1000 bp of the mutant \textit{nit-3} allele failed,
presumably due to such extensive mutagenesis of the nucleotide sequence, that
hybridization failed to occur with the wild-type primers. Figure 29 and 30 show partial
nucleotide sequences of the 5'-' and 3'-'-ends, respectively, of the nit-3 gene in the RIP22A
mutant strain as compared to the wild-type sequence. Two noteworthy mutations include
a change in the translational start codon to a isoleucine codon (Figure 28) and the
presence of a new EcoRI restriction site in the 3'-'-end of the gene that correlates well
with the change in restriction pattern as shown by Southern analysis (Figure 28). In all,
a 790 and a 206 nucleotide sequence were analyzed in the 5'-' (Figure 31) and 3'-'-region
(Figure 32), respectively. A possible nitrate reductase protein in the RIP22A strain was
tested for by Western blot experiments, which showed that no protein was detected by
the anti-NIT3 antibody (Figure 33).

Transformation of the RIP22A Mutant Strain

The primary goal for generating a strain with a heavily damaged nit-3 mutant
strain was to prevent homologous recombination with the nit-3 gene during
transformation. Thus, it was necessary to determine whether the new RIP22A mutant
strain would be a suitable host for transformation assays. The same deletion subclones
that were used in the previous studies described above with the nit-3 mutant strain
14789A were transformed into the RIP22A strain and selected on nitrate medium. As
shown in Figure 34, the subclones which were missing any part of the coding region
could not transform the new nit-3 mutant strain, suggesting that homologous
recombination had been totally eliminated with this mutant strain. Moreover, RIP22A
pN3Δ3UT transformants were found to have a significantly lower level of nitrate
reductase activity than wild-type (Figure 35). Interestingly, this deletion lacks the entire 3'-nontranslated region of the nit-3 mRNA. Thus, these results suggest that specific sequences 3' of the Neurospora nit-3 gene might be important for its expression.

Discussion

A better understanding of the structural and functional properties of nitrate reductase has been gained from biochemical and genetic studies of conventional nit-3 mutants. The utilization of chlorate, a toxic analog of nitrate in wild-type Neurospora, has been valuable to select for nitrate assimilation deficient mutants. In addition, the ability to independently assay the individual cofactor-binding domains of nitrate reductase for redox activity via artificial electron donors and acceptors has greatly enhanced the phenotypic characterization of nitrate reductase mutants (Tomsett and Garrett 1979; 1981). These earlier studies, however, involved much tedious work and yet, only provided a crude estimate of the location or type of particular mutation. With the advent of the recombinant DNA technology and, in particular, the polymerase chain reaction (PCR), it has now become possible to precisely and fairly rapidly determine the nature of mutant alleles at the molecular level.

Three conventional nit-3 mutant alleles, V1M16, V1M4 and 14789A, which had previously been shown to lack all or part of the nitrate reductase partial activities, were PCR-amplified and cloned. Nucleotide sequencing revealed that the mutant allele, V1M4, contained a single base change from T to A at position +822 (+1 position is defined at the first transcriptional start site). This changed a leucine codon (TTA) into a TAA nonsense codon early in the coding region (Figure 19), which would encode a
truncated protein of 237 amino acid residues with an estimated molecular weight of 26 kDa. Single base deletions were found for the mutant strains, 14789A and V1M16, at positions +2331 and +2858, respectively, causing a shift in the reading frame (Figure 19). The nearest in-frame stop codons for 14789A and V1M16 occur at positions +2713 and +3187, respectively. Mutant proteins of 760 amino acid residues for 14789A and of 918 amino acids for V1M16 would be expected. These results are in very good agreement with data from previous studies which had shown that nit-3 allele V1M4 possessed a null phenotype whereas alleles 14789A and V1M16 exhibited partial activities for the heme- and molybdenum-binding domains but not for the flavin-binding domain. These results demonstrate that despite a single base mutation in a cofactor-binding domain, the integrity of the protein remains intact since individual domains can retain function independently of each other. Thus, nitrate reductase provides an excellent system in which to undertake structure-function studies of a complex enzyme with multiple redox centers.

As demonstrated in Chapter III, the nit-3 gene and protein are highly regulated with respect to nutritional conditions. However, previous indirect studies with nit-3 mutants generated by Tomsett and Garrett (1981) found that some of these mutants, including V1M16 and 14789A, displayed an altered mode of regulation. These mutants seemed not to require induction as evidenced by the detection of nitrate reductase partial activities in the presence and absence of the nitrate. These data, subsequently, led Tomsett and Garrett (1981) to postulate that Neurospora nitrate reductase may regulate its own synthesis in much the same way that Aspergillus nitrate reductase does (Cove
1976). Therefore, it was of interest to more directly determine whether control of nitrate reductase synthesis is altered in the strains V1M16 and 14789A.

Western analyses of strains V1M16 and 14789A grown under both nitrogen repressed and nitrogen derepressed, nitrate induced conditions revealed the constitutive presence of the nitrate reductase protein in both mutants (Figure 22). An autoregulatory model was originally proposed by Cove (1976) for Aspergillus nitrate reductase and later adapted for Neurospora nitrate reductase. This model suggests that nitrate reductase inhibits the activity of the pathway-specific nitrogen regulatory protein (i.e., NIT4, in the case of Neurospora) and thus, its own synthesis via a protein-protein interaction in the absence of nitrate. When nitrate is present, nitrate reductase is visualized to bind nitrate, releasing the NIT4 protein, which can then turn on the synthesis of nitrate reductase. Thus, an alteration in the structure of the protein as might be expected with both V1M16 and 14789A could, indeed, affect an interface required for protein-protein interactions.

The precise identification of the mutation in the nit-3 mutant strain 14789A provided an excellent opportunity to test whether a significant degree of homologous recombination occurred with the nit-3 gene, as earlier suspected (see Chapter IV). Transformations with nested deletion nit-3 subclones, which covered the mutation site in 14789A, all restored the nit-3 mutant host strain's ability to grow in nitrate regardless of the deletion size. In fact, one deleted nit-3 gene which lacked coding sequence for an entire domain (pN3Δ620; Figure 23) still transformed the 14789A mutant, strongly suggesting that homologous recombination was responsible. Furthermore, the capability of the transformants to grow in nitrate was demonstrated to be due to the presence of a
functional nitrate reductase. Convincing evidence that the \textit{nit-3} gene has a penchant for targeting to the resident locus was provided by demonstrating that the transformants have a full length nitrate reductase protein. Since neither the resident mutant gene nor the deleted transforming \textit{nit-3} subclones encode a full length protein, its presence implies that homologous recombination did indeed occur.

In order to pursue structure-function studies on nitrate reductase, a \textit{nit-3} mutant host that did not allow targeting was needed. The ideal host strain would be one in which the entire \textit{nit-3} resident locus was missing. Using the information that the \textit{nit-3} gene targeted very well to its locus, attempts were made to construct such a strain by transforming wild-type spheroplasts with an almost totally deleted \textit{nit-3} subclone. However, because neither the number of copies integrating into a genome nor the chromosomal locations in which they integrate cannot be controlled, all of the \textit{nit-3} deletion strains generated had at least two copies of the partially deleted \textit{nit-3} gene (data not shown). Moreover, attempts to cross out the extra copies did not succeed. Therefore, a second approach, which utilized the recently discovered "RIP" phenomenon, was employed to produce a suitable host strain that was heavily damaged at the \textit{nit-3} locus.

The repeat-induced point mutation, or RIP, process was first reported by Selker \textit{et al.} (1987). Duplicated sequences, either unlinked or linked, in the \textit{Neurospora} genome were found to be unstable when put through a cross, and occurred premeiotically and immediately after fertilization. Analysis of single-copy transformants demonstrated that mere integration of a DNA segment into the genome did not provoke the RIP
process (Selker and Garrett 1988), suggesting that *Neurospora* may possess a mechanism for detecting and altering sequence duplications. A change in restriction patterns, which is indicative of RIP, was found to exclusively result from transition point mutations in which G:C pairs are converted to A:T pairs (Selker 1990a). *Neurospora* sequences which have undergone RIP were found to be heavily methylated on many cytosine residues in the duplicated regions and this methylation appeared to be stable through several rounds of DNA replication. Moreover, it seemed that although sequence duplications might initially trigger RIP, they were not necessary for methylation of RIP-mutated sequences (Selker 1990a). Therefore, it has been postulated that the presence of the point mutations makes the sequences susceptible to DNA methylation (Selker 1990b).

The use of the RIP phenomenon to generate a competent host strain with a severely damaged *nit-3* resident gene proved to be successful. Detailed genetic and biochemical characterizations of the newly constructed mutant *nit-3* strain, RIP22A, conclusively showed that the *nit-3* gene had been severely damaged. Most importantly, the extensive damage incurred by the resident *nit-3* locus of strain RIP22A prevented homologous recombination with transforming *nit-3* genes. Thus, RIP22A is a suitable host for transformation assays.

Sequence analysis of the RIP22A mutant *nit-3* allele revealed a significant number of point mutations throughout the duplicated region. With respect to the coding strand, at the 5'-end, 42% of guanosines (71 of 171) and 20% of cytidines (60 of 307) were converted exclusively to adenosine and thymidine residues, respectively, in a 790
nucleotide stretch (Figure 31). These mutational changes caused the modification of 104 amino acid codons of which 71 altered the amino acid residue, including the creation of 8 stop codons. Likewise, within a 206 nucleotide stretch at the 3'-end, examination of the coding strand showed 13 of 37 guanosines (35%) and 7 of 74 cytidines (9%) to be mutated, changing 14 codons and 13 amino acid residues including one to a stop codon (Figure 32). Moreover, 12 of the 61 bases in the intron had been altered, including those at the 5'- and 3'-splice sites and the internal branch sequence. Interestingly, it appeared that there was a preference for G-to-A over C-to-T changes in one strand, as evidenced by the higher number of mutated guanosine residues at the 5'- and 3'-ends (Figure 32). This apparently nonrandom difference in mutagenesis by the RIP process of one strand over another has also been observed with other Neurospora genes (Camberari et al. 1989; Selker 1990a; Jarai and Marzluf 1991b), and in fact, an extreme example of strand selectivity was shown to occur with a fragment of the nmr-1 gene in which G to A changes were exclusively present on only one strand (Jarai and Marzluf 1991b). From these observations, it has been suggested that the RIP machinery works in a processive manner and involves a putative DNA-cytosine deaminase which catalyzes the conversion of cytosine residues to uracil residues (Selker 1990a).

Additional sequence analysis of the nit-3 mutant allele, RIP22A, revealed that the base transitions do not seem to occur in an arbitrary manner, but are specific for cytidine residues which are immediately followed by an adenosine base (Selker 1990a). In contrast, cytidines that are 5' to another cytidine seldom undergo point mutations (Camberari et al. 1989) regardless of whether the duplicated sequences are linked or not.
When the coding strand was examined for the frequency of CpA, CpT, CpG and CpC dinucleotides lost due to the RIP process in the nit-3 mutant allele RIP224, it was found that 38% of cytidines in CpA sequences were changed while merely 20%, 18% and 3% in CpG, CpT and CpC, respectively, were altered at the 5'-end. Similarly, 33%, 8%, 0%, and 0% of CpA, CpT, CpG and CpC, respectively, were altered at the 3'-end. In addition, studies with other Neurospora genes which had gone through one round of RIP have shown that the mutations seem to be clustered (Camberari et al. 1989; Jarai and Marzluf 1991b). However, point mutations in RIP224 appear to be scattered throughout the 790 nucleotide region examined, possibly as a result of two cycles of the RIP process. Because only a very short segment of the 3'-end was analyzed, the distribution of the mutations could not be determined. Nevertheless, the RIP process has been shown to occur through approximately six generations, after which the duplicated DNA sequences have mutated so badly that they are no longer recognizable as comparable sequences (Camberari et al. 1991). Moreover, studies have revealed that linked sequence duplications undergo a greater divergence in sequence before becoming resistant to RIP mutagenesis than unlinked duplicated sequences. Therefore, divergence of the homologous regions as well as the loss of the preferred cytosine dinucleotide sequences (i.e., CpA) are significant for conferring resistance to the RIP process.

Although the RIP phenomenon has been extensively studied in Neurospora, it has also been demonstrated to occur in a few other fungi including Ascobolus and Gibberella but not in Saccharomyces, Podospora or Aspergillus (Selker 1990a). The use of RIP is not only valuable to create host strains for transformation studies but also to assess the
function of cloned genes that have no known mutants (Marathe et al. 1990). The evolutionary significance of RIP has been implicated to be important for maintaining a compact genome by modifying redundant sequences. The RIP process may be important in maintaining the stability of the genomic organization of *Neurospora* (Selker 1990a).

An important means of carrying out structure-function studies is by specific mutagenesis of a cloned gene *in vitro* whose function is assessed *in vivo* by transformation into a competent host strain. Interpretation of these transformation assays, however, is often complicated by targeting of the transforming DNA and complementation of the mutant protein encoded by the resident locus. The RIP process was used to generate a mutant strain, RIP224, shown to be extensively mutated throughout its *nit-3* locus, and therefore, will serve as a suitable host for transformation studies.
CHAPTER VI
STRUCTURE AND FUNCTION STUDIES OF THE
HEME-BINDING DOMAIN OF NITRATE REDUCTASE

Introduction

In order to gain a better understanding of the intramolecular electron transport mechanism which enables nitrate reductase to be a highly efficient enzyme, a detailed knowledge concerning the structural and functional features of each cofactor-binding domain is essential. The ideal approach to study structure-function relationships would be to determine the crystal structure of the complete nitrate reductase protein. Unfortunately, no such data exist; although primary sequences of nitrate reductases from a variety of organisms have been found to be highly homologous, particularly within the binding domains for the prosthetic groups: molybdenum, heme and FAD. Each domain also has strong homology with smaller, functionally unrelated redox proteins, such as sulfite oxidase, cytochrome b₅ and cytochrome b₅ reductase, which bind one or more of the same cofactors as nitrate reductase. These conserved regions have, thus, facilitated the identification of potentially critical amino acid residues for each domain, as discussed in Chapter IV.
Of the three cofactor-binding domains in nitrate reductase, the heme-containing domain affords the best opportunity to examine regional structure and function relationships not only because of its significant sequence similarities with the cytochrome b₅ family, but more importantly, due to the determination of the three-dimensional structure for bovine liver cytochrome b₅ (Mathews et al. 1972). As a result of its homology to the cytochrome b₅ proteins, the heme domain has the best defined boundaries, encompassing only about 70 amino acid residues, in sharp contrast to the larger sizes of the molybdenum and FAD domains which possess at least 400 and 260 amino acids, respectively (Figure 15). In addition, polyclonal antibodies produced specifically against this domain (see Chapter V) have proven useful in examining the effect of structural changes on protein stability. Consequently, the combination of a small domain size, extensive homology to a physiologically similar redox protein of known crystal structure, and available polyclonal antibodies that are selective for the *Neurospora* nitrate reductase heme domain, provides a straightforward strategy to molecularly manipulate and assess this domain for important amino acid residues.

Structural studies that focused on the *Neurospora* nitrate reductase heme-binding domain were undertaken by utilizing the crystal structure of cytochrome b₅ as a model. Specific amino acids, which were previously hypothesized to be of possible functional significance in this domain, were altered by site-directed mutagenesis. These mutants were evaluated *in vivo* by transformation assays conducted with the newly generated *nit-3* mutant strain RIP22A (Chapter V). In addition, anti-NIT3 antibodies permitted the direct examination of previously isolated *nit-3* amber nonsense strains which showed varying
levels of nitrate reductase activity upon suppression with the suppressor strain *Ssu-1* (Perrine and Marzluf 1986). Since the suppressor inserted a tyrosine residue at the amber mutation site, it was of interest to determine the approximate location of each of the *nit-3* amber mutants and to verify whether the amount of nitrate reductase protein produced after suppression of these strains correlated with earlier nitrate reductase assays.

**Results**

**Functional Analysis of the Heme-binding Domain by Site-directed Mutagenesis**

The extensive homology of the heme-binding domain of nitrate reductase with cytochrome b₅ suggested that they had similar three dimensional structures. Hence, the crystal structure of bovine liver cytochrome b₅ was used as a model to identify potentially significant amino acids for functional analysis. Figure 36 gives the tertiary structure of bovine liver cytochrome b₅ and indicates the position and nature of each amino acid that I altered in the *Neurospora* nitrate reductase heme-binding domain. The amino acids were selected on the basis of their conservation with cytochrome b₅ and their importance to functional integrity as implicated by previous studies (Lê and Lederer 1983). Since the variable Glu-633 residue was not expected to produce a notable effect on protein function, it was mutated for use as a control. With only two exceptions, the amino acid residues were changed to the neutral residue, alanine (Table 4), by site-directed mutagenesis. One mutation, which unexpectedly changed a conserved Trp-635 residue to Phe, was obtained by serendipity. Mutagenesis was carried out with a *nit-3* *SmaI*-*StyI* cDNA subclone using mutagenic oligonucleotide primers, and the mutagenized regions
were sequenced to confirm that the desired substitutions had been achieved. An \textit{XhoI-SstI} fragment encompassing the mutagenized site was used to replace the analogous region in a \textit{nit-3} subclone which was identical to the wild-type \textit{nit-3} gene except that it lacked the one of the NIT2 protein binding sites.

To ascertain the functional effect of each of the mutations in the heme-binding domain, the mutagenized \textit{nit-3} genes were transformed into the \textit{nit-3} mutant host strain RIP22A and tested for their ability to grow in nitrate medium. As a control, the wild-type \textit{nit-3} gene (containing two of three NIT2 sites) was also transformed into the identical host strain. Table 4 shows the transformation frequency for each transformed mutant gene. Not surprisingly, mutations occurring within the proximity of the heme-binding pocket generally led to significant reductions in transformation frequency as compared to that for the wild-type gene. The conservative change of Trp-635 to Phe did not affect protein function since a large number of transformants were obtained (Table 4). Alterations of two nonconserved amino acids that are located away from the heme-binding site (\textit{i.e.}, Glu-633 and Ser-701) resulted in different transformation frequencies. That is, changing Glu-633 to alanine did not appear to affect function as shown by transformation frequencies similar to wild-type. Conversely, the mutation of Ser-701, which is localized in the putative hinge region between the flavin and heme domains, significantly decrease the number of transformants.

\textbf{Southern Analysis of the Heme Mutants}

Since it was desired to assess the effect of these mutations on protein structure and function in detail, homocaryons were prepared for each heme mutant. Certain \textit{nit-3}
mutants which failed to produce transformants (H675A, I659G, S676A; Table 4) on nitrate medium, were rescued by growing the transformed fungal cells in nonselective medium and then randomly selecting a number of colonies. Those that possessed one or more copies of the transforming DNA as determined by Southern analysis (data not shown) were, subsequently, used to isolate homocaryons. Genomic DNA was isolated from several individual homocaryotic colonies for each of the mutants, digested with EcoRI and analyzed by Southern blot experiments. Digestion with EcoRI gave a characteristic restriction pattern for the RIP22A host strain when probed with a 1.5 kb HindIII-BglII fragment, which permitted the determination of copy number of transformed DNA in each homocaryon. Figures 37, 38 and 39 present the results of the Southern analysis. Several colonies that contained either single copies of manipulated nit-3 genes (mutants H652A, F648A, W635F, E633A, and S694A) or multiple copies for the nonfunctional mutants (H675A, I659G and S676A), were used for further analysis.

Phenotypic Characterization of the Heme Mutants

For each heme mutant, partial and complete enzyme activity assays were carried out on crude cellular extracts prepared from nitrogen repressed and nitrogen derepressed, nitrate induced mycelia of at least three independent homocaryons. The flavin and flavin-heme domains were assayed by using the artificial electron acceptors, ferricyanide and cytochrome c, respectively. Reduced methyl viologen was utilized as an artificial electron donor to measure the combined activities of the molybdenum and heme domains. The crude extracts were subjected to ultracentrifugation to obtain purer cytoplasmic
samples. However, cytochrome spectra of the heme domain were not obtained due to interference with other respiratory proteins, as evidenced by the appearance of characteristic Soret bands in nitrogen repressed wild-type extracts (i.e., the negative control).

The levels of nitrate reductase activity and its associated partial activities for each heme mutation are shown in Table 5. Consistent with the transformation studies, mutants H675A, I659G and S676A lacked any nitrate reductase activity. Mutants H652A, F671A, E633A, W635F and S694A, which transformed the Neurospora nit-3 mutant strain at varying frequencies, were all shown to have significant nitrate reductase activity. Thus, these mutants are presumed to possess functional nitrate reductases. The reason for the lower number of transformants observed in mutants F671A and S694A is unknown, although the quality and concentration of DNA, which is difficult to assess precisely, may have contributing effects. Curiously, both mutants F648A and W635F displayed some nitrate reductase activity under nitrogen repressed conditions, suggesting an aberrant mode of regulation. Figure 40 graphically presents the levels of nitrate reductase activity observed in these mutants.

In comparison to the wild-type transformant, the levels of methyl viologen-nitrate reductase partial activity were generally higher than wild-type among those mutants that were able to reduce nitrate (Table 5 and Figure 41). Previous studies have demonstrated that partial denaturation of wild-type nitrate reductase increased the activity for the collective molybdenum and heme domains by as much as 8-fold (Garrett and Nason 1969). Although such a large difference in activity was not observed here, partial
denaturation of the protein conformation may have occurred with these heme mutants, resulting in the enhanced accessibility of methyl viologen to its active site. This possibility is suggested by the observed higher levels of methyl viologen-nitrate reductase partial activity. Those mutants that lacked complete nitrate reductase activity also did not display any partial activity in the assay for the coupled molybdenum and heme domains, as expected.

The levels of partial activities for the flavin and flavin-heme domains differed significantly among the mutants (Table 5). Since crude cellular extracts were used, the variable levels of activity in these mutants may be attributed to basal levels of NADPH-dependent cytochrome c reductase activity contributed by respiratory enzymes as well as by NADPH-nitrite reductase (Garrett and Tomsett 1981). Those mutants which displayed neither partial activity for the molybdenum and heme domains nor nitrate reductase activity, were found to have lower levels of activity for the flavin and flavin-heme domains under induced conditions than found in the wild-type transformant or wild-type strain 74A even under repressed conditions (Table 5). This result suggests that these mutants also lack these domain activities. The partial activities of the nitrate-utilizing heme mutants, H652A, F671A, E633A, W635F and S694A for the flavin-heme domains appeared to be inducible and comparable to wild-type under the contrasting nitrogen conditions (Table 5). Presumably, the substituted amino acids did not have a deleterious effect on the activities of the flavin or heme domains in these mutants. The relatively higher partial activity levels observed for F671A and W635F under nitrogen repressed conditions probably results from the partial constitutive presence of the nitrate reductase
protein, as demonstrated with the nitrate reductase assay. The results of the assay of the flavin domain alone for each of the heme mutants were generally consistent with the previous enzyme activity data. For those mutants which so far had shown no partial activity or nitrate reductase activity, the enzyme levels for the flavin domain did not seem to change significantly from repressed to induced conditions (Table 5). On the other hand, mutants which did exhibit nitrate reductase activity also displayed a higher level of flavin partial activity under inducing conditions. The wild-type transformant showed a lower increase in this partial activity upon induction for some unknown reason (Table 5). In summary, the results of these enzyme assays are consistent with the in vivo function of the mutant protein as determined by transformation.

Western Analysis of the Heme Mutants

To investigate whether each introduced mutation affected the stability of the entire nitrate reductase protein or altered its regulation, crude cellular extracts were prepared from mycelia that had been grown under both nitrogen repressed and nitrogen derepressed, nitrate induced conditions for each of the heme mutants as well as for wild-type strain 74A and for the wild-type nit-3 gene transformant. Identical protein samples were electrophoresed in two 7.5% denaturing polyacrylamide gels. One gel was stained for protein with Coomassie Brilliant blue, and the other was probed with the anti-NIT3 antibody. Western analysis of nitrate reductase in the heme mutants, H652A, F671A, E633A, W635F and S694A, showed variable levels of the protein (Figure 42). As compared to the wild-type nitrate reductase protein, the abundance of the protein differed among the various heme mutants. The amino acid substitutions in the mutant proteins
may have resulted in an increased susceptibility to proteolysis. Moreover, three mutants, F671A, W635A and H675A, appear to display an aberrant mode of regulation as evidenced by the presence of the nitrate reductase protein in both repressed and derepressed extracts (Figure 42A & B). Curiously, the heme mutant H675A which exhibited neither nitrate reductase activity nor transformation of a nit-3 Neurospora mutant strain to prototrophy, possessed the nitrate reductase protein, albeit at a lower level than wild-type. A mutation which changed another histidine residue, His-652 (mutant H652A), that, along with H675A is thought to function in directly binding the heme-iron, was present under normal regulatory conditions (Figure 42D). This suggests strongly that the mutations of His-652 and His-675 do not significantly affect the stability of the protein and thus, must affect its function. Two other mutants, I659G and S676A, which also gave no transformants or catalytic activity, lacked any nitrate reductase protein, presumably due to its high lability (Figure 42B & C).

**Heme-Binding Ability of Two Histidine Mutants**

The results obtained from Western analysis of the two histidine mutants, H652A and H675A, as well as the potential importance of these residues in protein function (Mathews et al. 1972) were the impetus to determine whether these mutant proteins retained their ability to bind heme. Crude cellular extracts were prepared from mycelia that had been grown under both nitrogen repressed and nitrogen derepressed, nitrate induced conditions for the two histidine mutants as well as for the wild-type strain 74A. The samples were run in duplicate on two 7% non-denaturing polyacrylamide gels at 4°C. Beta-galactosidase (540 kDa) was used as a size marker for comparison with the nitrate
reductase protein (216 kDa). One gel was stained for protein with Coomassie Brilliant blue to insure that equal amounts of protein extract had been loaded into each lane (Figure 43). The other gel was stained for heme with 0.2% benzidine dihydrochloride-hydrogen peroxide. Figure 43 reveals that heme mutant H652A, which had previously been demonstrated to produce a stable and functional nitrate reductase protein, was able to bind the heme moiety, albeit at a much lower affinity than the wild-type nitrate reductase protein. The other heme mutant, H675A, which was shown to produce a stable but nonfunctional protein did not bind heme to any discernible level. Thus, it appears that although both histidine residues are important, one (His-675) has a much greater effect upon heme binding than the other (His-652).

**Western Analysis of nit-3 Amber Nonsense Mutant Strains**

Crude cellular extracts of four nit-3 amber nonsense mutants and their suppressed mutant strains (alleles KGP1222, KGP0213, KGP026, KGP1211) were prepared from mycelia that had been grown under both nitrogen repressed and nitrogen derepressed, nitrate induced conditions. The samples were loaded in duplicate onto 7.5% denaturing polyacrylamide gels along with extracts from wild-type strain 74A and, in some cases, nit-3 mutant strain 14789A (see Chapter V) as controls. One gel was stained for protein with Coomassie Brilliant blue to show that equal amounts of sample had been loaded per lane. The other gel was electroblotted onto two layers of nitrocellulose filters that were then probed with the anti-NIT3 antibody, with detection via a secondary antibody conjugated to horseradish peroxidase. The results shown in Figure 44 imply that these nit-3 mutant alleles possess the amber nonsense mutation at four different locations.
Furthermore, the levels of expression of nitrate reductase protein in the suppressor strains are in good agreement with prior nitrate reductase activity and growth studies (Perrine and Marzluf 1986). An additional smaller protein band, about 85-90 kDa, was observed in all but one of the suppressor mutant strains. This smaller protein may result from enzyme instability due to the insertion by the suppressor of a tyrosine residue at a site normally occupied by other amino acids, and thereby, resulting in an altered enzyme that may easily undergo partial degradation. Interestingly, the amber nonsense mutant proteins that were detected (i.e., alleles KGP1222, KGP1211 and KGP026) appear to be constitutive; that is, have an altered control. Upon suppression, however, the normal mode of regulation is restored. Such an altered control of nitrate reductase has also been observed with other nit-3 mutants (e.g., V1M16 and 14789A; Chapter V).

Discussion

Since nitrate reductase possesses discrete binding domains for three prosthetic groups that collectively catalyze the efficient reduction of nitrate, its structural properties are of particular interest. A complete molecular understanding of electron transfer and domain-domain interactions within nitrate reductase requires an analysis of the entire protein; however, detailed structural investigation of the nitrate reductase protein has historically been restricted owing to its low abundance and high lability. Although limited proteolysis of spinach nitrate reductase has been found to isolate active forms of the individual domains as determined by partial enzyme activity assays, the accumulation of a large quantity of the domains after proteolytic cleavage for functional analysis is difficult (Kubo et al. 1988). Therefore, the availability of crystal structures for other
well-studied redox proteins that bind the same cofactors as does nitrate reductase has been extremely valuable in the study of nitrate reductase protein structure. Moreover, the use of recombinant DNA technology and, in particular, site-directed mutagenesis, has allowed for a more direct approach in the examination of structure and function relationships that have been proposed for nitrate reductase.

Three dimensional studies of the cytochrome b₅ protein (Mathews et al. 1972) had shown that the heme-binding domain is cylindrical and consists of six short α-helical regions with five interspersed β-sheets which divide the protein into two uneven fragments. Using the cytochrome b₅ numbering, the larger entity whose linear sequence is from residue 21 to 78 (Figure 36) is involved in binding of the heme molecule and thereby, functions in electron transport. The smaller fragment, contained within residues 3-20 and 78-87, appears to have a more structural role although the region is also highly conserved. There are 14 amino acids that form a binding crevice and come in contact with the heme molecule, and of these, eleven are hydrophobic. Three polar amino acids, two histidines (His 39 and His 63) and a serine residue (Ser 64), which are considered to be the most functionally significant residues of the domain, coordinate the binding of heme within the pocket. The hydroxyl group of the serine residue is proposed to form hydrogen bonds with the heme group at two different sites while the two histidine residues are believed to directly hold the heme molecule in the pocket through their ε-NH groups (Mathews 1972). The precise alignment of the heme molecule in the crevice such that one side preferentially faces one histidine residue and not the other is facilitated by a conserved phenylalanine residue, Phe 58, which is postulated to pack tightly against
His 63 in a planar manner and stabilize its orientation via a hydrogen bond (Mathews 1980). From primary sequence comparisons of a number of heme-containing proteins that are functionally dissimilar such as nitrate reductase, sulfite oxidase and cytochrome b₅, Guiard and Lederer (1979) have proposed a protein structure called the cytochrome b₅ fold. In addition to the aforementioned histidine and phenylalanine residues, the cytochrome fold contains the invariant sequence, Pro 40, Gly 41, Gly 42, that is believed to permit a 180° directional change in the protein and to stabilize His 39 via a hydrogen bond with the peptidyl carbonyl group of Gly 42.

To take advantage of the detailed structural information available on the homologous cytochrome b₅ family, the heme-binding domain of *Neurospora* nitrate reductase was selected for protein structure and function studies. The primary sequences of the cytochrome b₅ protein family have previously been shown to be significantly similar to that of the heme-binding domain of nitrate reductase. Utilizing both the crystal structure of the calf liver cytochrome b₅ (resolved to 2.0 Å) as a model of the heme-binding domain of *Neurospora* nitrate reductase and the invariant amino acids found in the nitrate reductase heme domains and the cytochrome b₅ proteins, specific amino acid residues were mutated and their effects assessed *in vivo*.

Several lines of evidence demonstrate that the two potentially significant histidine residues, His 652 and His 675, of *Neurospora* nitrate reductase do not equally contribute to the binding of the heme group *in vivo*. Functional analysis by transformation assays of the histidine mutants demonstrated that the frequency of transformation was much greater with the H652A mutant than with the H675A mutant (Table 4). Partial and
complete enzyme activity assays, furthermore, revealed that only one histidine mutant (H652A) displayed any nitrate reductase or partial enzyme activity (Table 5). Western blot experiments showed that the mutant nitrate reductase proteins were not stable in these two mutants although there appears to be less protein for the H675A mutant (Figure 42A) and its regulation was altered as evidenced by its presence in both nitrogen repressed and nitrogen derepressed, nitrate induced extracts (Figure 44A). Finally, direct analysis of heme in these mutant proteins revealed that both of these histidines appear to, indeed, play an integral role in heme binding. However, the mutant protein substituted for His-652 still seems to allow for some coordination of heme, though weakly as compared to wild-type nitrate reductase. In contrast, substitution of His-675 apparently abolishes the heme binding capability of the resulting mutant nitrate reductase protein (Figure 43). Moreover, the harsh electrophoretic conditions to which the mutant H652A protein was subjected may also account for even less apparent heme content than present in the native enzyme.

The significance of these two histidine residues in the binding of the heme and thus, function of the protein, have been demonstrated through extensive studies with other proteins. His 63, which corresponds to His-681 of Neurospora nitrate reductase, was changed to alanine in a mammalian cytochrome b₅ protein and expressed in E. coli. This mutant cytochrome b₅ protein was unable to incorporate heme and moreover, did not reconstitute exogenous heme after protein purification (Beck von Bodman et al. 1987). Replacement of the histidine with methionine, however, resulted in a functional protein although it was revealed that the sulfur group of methionine did not bind to the
heme iron. Instead, the extra volume filled by the methionine side chain was postulated to exclude water from the heme ring and thereby, promote its incorporation. More recently, a nitrate reductase mutant of tobacco was shown to have one of the histidine residues altered to an asparagine, producing a nonfunctional nitrate reductase that could not catalyze any of the partial enzyme reactions that required the heme domain (Pouteau et al. 1989; Crawford and Campbell 1990).

In addition to the two histidine residues described above, I also carried out mutagenesis to replace other amino acid residues around or beyond the heme-binding pocket. Consistent with the in vivo assays for function in Neurospora, the levels of enzyme activity revealed that mutations of certain amino acids caused only minor effects, whereas other substitutions had a very detrimental effect on protein function. Alterations of residues which are located outside the binding crevice produced proteins with nearly wild-type transformation efficiency and enzyme activity. The decrease in transformation frequency observed with the S694A mutant may be due to conformational changes in the protein, resulting in a less stable but still functional protein as evidenced from Western analysis (Figure 43) and from partial enzyme activity assays (Table 5).

Mutations that changed amino acids around the heme pocket revealed a more dramatic effect on the function of the heme domain. The conversion of an isoleucine residue (Ile-659) to glycine resulted in a complete lack of the nitrate reductase protein as shown by a lack of transformants (Table 4), no full or partial enzyme activities (Table 5) and the absence of a stable protein (I659G; Figure 44B). The Ile-659 is well conserved with regard to its nonpolar nature, and is postulated to be significant in
maintaining the hydrophobicity of the heme-binding domain by lying very close His-652 (Mathews et al. 1972). The substitution of glycine, which has no significant side chain, for Ile-659 may disturb the protein conformation such that the heme is not bound properly. More importantly, Ile-659 appears to be part of an α-helix and its mutation to a glycine residue, which is not often found in α-helices, may disrupt this helix. Alterations of both the highly conserved Phe-671 and Ser-676 residues, which were previously hypothesized to be important in maintaining the heme moiety in the hydrophobic crevice, were found to have varying influences on nitrate reductase functionality. Although the mutation of Phe-671 resulted in a significant decrease in transformation frequency (Table 4), viable homocaryons harboring the mutagenized nit-3 gene were shown to have significant levels of nitrate reductase and partial enzyme activities. Furthermore, Western analysis showed that the mutant nitrate reductase protein appeared to be as stable as the wild-type nitrate reductase protein. In contrast, mutation of the Ser-676 residue, which is adjacent to one of the heme-binding histidines (His-675), resulted in a complete loss of transformability, enzyme activity and protein stability. This amino acid has been postulated to be significant in the coordination of the heme moiety within the pocket via two hydrogen bonds through its hydroxyl side chain and its peptidyl amide group (Mathews 1985). Even a conservative amino acid change from serine to alanine apparently causes destabilization of the entire protein. In contrast, mutagenesis of the analogous serine residue, Ser 64, in an E. coli expressed cytochrome b₅ protein and its analysis by x-ray crystallography demonstrated that despite the loss of one hydrogen bond (via the hydroxyl side group) the proper orientation of the heme
was still maintained; the mutant cytochrome b₅ also displays a characteristic low reduction potential (Funk et al. 1990), suggesting that one hydrogen bond is sufficient for heme binding. The disparity concerning the serine residue observed between this study and that of Funk et al. (1990) may, in part, be due to the fact that since nitrate reductase contains multiple redox centers, the orientation and proximity of the other prosthetic groups relative to the heme moiety cannot be ignored. Thus, changes in nitrate reductase conformation may account for the observed instability of the mutant protein containing the serine to alanine mutation.

The constitutive presence of the nitrate reductase protein under the nitrogen repressed condition in heme mutants F671A, W635A and H675A may be due to an altered mode of regulation which has also been observed with other nit-3 mutant alleles such as V1M16 and 14789A (see Chapter V). However, unlike the alleles V1M16 and 14789A, which have single base deletions, thus creating frameshifts leading to completely defective proteins, the heme mutants all cause only single amino acid substitutions. Consequently, these directed mutations appear to affect the structural integrity of the protein, leading to the loss of normal regulation as well as function in the case of heme mutant H675A.

The availability of antibodies specific for the nitrate reductase protein has permitted the analysis of previously isolated nit-3 amber mutants and their corresponding suppressor strains. These amber nonsense mutants were shown to lack any nitrate reductase activity. However, upon suppression, enzyme activity was restored at varying levels ranging from approximately 10% to 40% of wild-type nitrate reductase activity
(Perrine and Marzluf 1986). Western analysis of the nitrate reductase protein in these mutants and their corresponding suppressor strains have found that the mutations do, indeed, occur at different sites in the nitrate reductase protein. Size estimates of the mutant proteins have revealed that allele KGP1222 probably is mutated in the heme-binding domain, resulting in a protein of 75-80 kDa (Figure 44), while the amber nonsense mutation in KGP0213 lies in the molybdenum domain since no protein could be detected with the heme domain-specific anti-NIT3 polyclonal antibody (Figure 44). The amber mutations of nit-3 mutant alleles KGP026 and KGP1211 appear to occur at two different sites in the FAD-binding domain (Figure 45). Moreover, my estimates of nitrate reductase protein content for each suppressed strain correlate well with previous nitrate reductase assays in which 40%, 30% 25% and 10% activity levels were observed for the mutants KGP026, KGP1222, KGP0213 and KGP1211, respectively.

The regulation of nitrate reductase synthesis has also been altered in the amber mutant strains KGP1222, KGP1211 and KGP026, as demonstrated by the presence of the protein in both nitrogen repressed and nitrogen derepressed, nitrate induced extracts. The constitutive presence of nitrate reductase in these amber nonsense mutant strains may be due to the dual regulatory and catalytic roles of the wild-type nitrate reductase protein (Tomsett and Garrett 1981).
In the filamentous fungus, *Neurospora crassa*, the *nit-3* gene encodes the enzyme nitrate reductase which catalyzes the initial reductive step in the ultimate conversion of nitrate to utilizable ammonia and thus, plays a major role in the assimilation of nitrate, an important source of the essential element, nitrogen. The expression of nitrate reductase is dependent upon the nitrogen source available in that it requires both the absence of a preferred nitrogen source such as glutamine or ammonia and the presence of nitrate. Furthermore, studies with regulatory mutants have strongly suggested that the control of nitrate reductase expression occurs at the transcriptional level (Fu and Marzluf 1987a). The accumulation and turnover of the *nit-3* mRNA and nitrate reductase enzyme have been demonstrated to occur rapidly in response to changes in the nutritional condition (Chapter III). Since the reduction of nitrate is an energetically expensive process, the rapid synthesis and turnover of the protein insure that cellular energy is not wasted when a preferred nitrogen source is available.

Sequence analysis and molecular characterization of the *nit-3* gene have furnished a significant amount of information on nitrate reductase (Chapter IV). Comparison of the deduced *nit*-3 protein sequence and nitrate reductase sequences from a variety of
organisms has led to a better understanding of the structure of nitrate reductase and the organization of its three functional domains for the molybdenum, heme and flavin cofactors. The conservation of specific amino acid residues among the nitrate reductases and with functionally similar but physiologically unrelated redox proteins such as cytochrome b₅ and cytochrome b₅ reductase has facilitated in the identification of regions potentially important for structure and function of the protein.

*Neurospora* is an amenable system in which to undertake structure and function studies due to ease of transformation and nonhomologous integration of transforming genes; thereby facilitating the interpretation of *in vivo* assays. However, unlike many *Neurospora* genes, *nit-3* was found to have a tendency to homologously recombine with its resident locus upon transformation (Chapter V). Thus, a new mutant *nit-3* allele was generated by the RIP process that precluded any homologous recombination and that created a suitable host strain for transformation assays needed for structure and function studies.

Taking advantage of the extensive sequence similarities between the heme-binding domain of nitrate reductase and cytochrome b₅, structure and function studies were carried out on this domain using the available crystal structure of bovine liver cytochrome b₅ as a model (Chapter VI). Site-directed mutagenesis of residues around and beyond the heme-binding pocket demonstrated that changes of amino acids in proximity of the crevice usually have a pronounced effect on function and stability of the nitrate reductase protein. Moreover, mutations of two histidines, which were suspected to be important in directly coordinating the heme moiety in the pocket, revealed that the
two residues did not have an equal role in heme binding. Interestingly, it appeared that His-675 was more directly involved in binding heme than His-652 (Figure 37), although both were found to be important for protein function as evidenced by transformation assays.

It should be noted, however, that the use of single redox center proteins like cytochrome b₅ as molecular models for one domain of a large polyfunctional protein like nitrate reductase must be interpreted with care due to the presence of other redox centers. One approach that could provide detailed information on one or more domains would be to express Neurospora nitrate reductase or parts of it in E. coli at a high level as has recently been done with the flavin domain of corn nitrate reductase, which was purified to homogeneity in a catalytically active form (Hyde and Campbell 1990). Expression of the heme-binding domain of Neurospora nitrate reductase would likewise provide an excellent means of obtaining a large amount of protein for crystallography and structure and function studies. In fact, the heme-binding domain has been expressed at a very high level in E. coli (Figure 21A); however, attempts so far to purify it were unsuccessful because most of the protein was present in inclusion bodies. More rigorous purification methods may eventually provide purified protein.

Studies on nitrate reductase have not only led to more information on nitrate metabolism but have also provided some practical applications. That is, although nitrate is an important source for nitrogen in plants, fungi, algae and bacteria, it is also a major environmental pollutant that comes primarily from decaying matter. One novel means to alleviate this problem has been to use immobilized nitrate and nitrite reductases to
catalytically remove nitrate from water (Mellor et al. 1992). This "bioreactor" is driven by an electrical current that results in the rapid and efficient conversion of nitrate to atmospheric nitrogen without producing residues. Thus, the efficient reductive reaction catalyzed by nitrate reductase has found application in combatting some of the growing environmental problems.

The work that has been presented here provides a firm basis on which further studies of nitrate reductase in Neurospora can be undertaken. A thorough investigation of the promoter sequences that are responsible for the highly inducible expression of nit-3 will be of interest. The mechanism of autogenous regulation by nitrate reductase may be addressed more fully now that the nit-4 gene has been molecularly characterized (Yuan et al. 1991). A detailed analysis of the structure and function of the cofactor-binding domains will be important in elucidating the mechanism by which nitrate reductase can efficiently catalyze nitrate reduction. In essence, a better understanding of nitrate reductase and its regulation will lead to a greater comprehension of both the function and control of the nitrogen metabolic circuit and the manner in which large proteins composed of multiple domains function.
Figure 1. The Nitrogen Metabolic Circuit of *Neurospora crassa*.
The effects of the regulatory genes, *nit-2*, *nit-4* and *nmr-1*, on the control of the structural genes are shown with plus and minus signs. The representative structural genes in the circuit and their products are as follows: *nit-3*, nitrate reductase; *nit-6*, nitrite reductase; *urc*, uricase; *alc*, allantoicase; *prt*, protease; *lao*, L-amino acid oxidase.
Figure 1
Figure 2. Northern Analysis of \textit{nit-3} Gene Expression in Wild-type \textit{Neurospora crassa}. \textit{nit-3} mRNA was isolated from mycelia that had been grown in nitrogen repressed or nitrogen derepressed, nitrate induced medium as described in Experimental Procedures. For hybridization, radiolabeled plasmids containing the \textit{nit-3} gene and the β-tubulin gene were used together to probe the blots. Lane 1 represents poly(A)$^+$ mRNA isolated from nitrogen repressed cells, and lane 2, from nitrogen derepressed, nitrate induced cells. The arrow identifies the 3.4 kb \textit{nit-3} transcript. The constitutively expressed β-tubulin mRNA, shown by the lower band, was used as an internal control to insure that equal amounts of mRNA were loaded and no excessive mRNA degradation had occurred.
Figure 3. Kinetics of Accumulation of the nit-3 Transcript.
Northern blot experiments were carried out on nit-3 mRNA for induction times of 0, 15, 30, 60, 120 and 180 minutes, and correspond to lanes 1, 2, 3, 4, 5 and 6 in the figure. A radiolabeled nit-3 plasmid was used to detect the message, and the constitutive β-tubulin transcript was used as an internal control.
Figure 3

1  2  3  4  5  6

← nit-3
← β-tubulin
Figure 4. Accumulation of Nitrate Reductase Enzyme Activity. Nitrate reductase assays were performed with crude cellular extracts as described in Experimental Procedures. The mycelia were induced for the same time periods as used in the Northern experiment in Figure 3. Specific activity is defined as nanomoles of nitrite produced per minute per milligram of protein, and the average standard deviation is ±1.66 U/mg.
Figure 4
Figure 5. Stability of the nit-3 Transcript Upon Nitrogen Repression. Northern analysis of nit-3 mRNA stability was conducted with RNA obtained from mycelia repressed for nitrogen following uniform nitrate induction. The probes were the same as in Figure 3. Lanes 1, 2, 3 and 4 represent 0, 5, 10 and 20 minutes, respectively, after repression. Due to the physical limitations, collection of repressed samples at shorter times was not feasible.
Figure 5

- nit-3
- β-tubulin
Figure 6. Determination of the Half-life of the $nit-3$ Message.
To calculate the half-life, each $nit-3$ mRNA sample was cut out of the Northern blot shown in Figure 5 and the amount of radioactivity counted. The corresponding internal standard was used to normalize the counts for each sample. The percentage of mRNA remaining was determined in comparison to the fully induced sample of Lane 1, Figure 5. The half-life of $nit-3$ mRNA is estimated to be about 5 minutes.
Figure 7. Turnover of the Nitrate Reductase Activity as Determined by Enzyme Assays. Nitrate reductase assays were performed on crude enzyme extracts of mycelia that had been repressed for the same periods of time as in the mRNA study of Figure 5. Each timepoint was assayed in duplicate. Specific activity is defined in the same way as in Figure 4, and the average standard deviation was calculated to be ±0.98 U/mg.
Figure 8. Sequencing Strategy and Restriction Map of the *nit-3* gene.
The restriction map of *nit-3* is shown on the top line. The large arrow indicates the size of the *nit-3* transcript as well as the direction of transcription, and the small crosshatched box displays the position of the single intron in *nit-3*. The smaller arrows represent the sequencing strategy via deletion subclones and oligonucleotide primers.
Figure 8
Figure 9. The Nucleotide Sequence of *nit-3* and Its Flanking Regions. Transcriptional start and stop sites are indicated by asterisks (*), and the long pyrimidine tract in the 5'-nontranslated region is overlined. The putative CAAT motif, TATA box element, polyadenylation signal, and G/T cluster are underlined while the NIT-2 protein binding sites are doubly underlined. For clarity, the single 61 bp intron is presented in lower case letters, and the deduced amino acid sequence is displayed directly below each corresponding codon. The first transcriptional site is assigned the +1 base position, and all nucleotide locations discussed in the text are relative to this. The upper and lower numerals on the right side of the figure represent the number of nucleotide bases and amino acid residues, respectively, which are present up to that point of the sequence.
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Figure 9 (continued)

TAT ACT TTG AGT AGA CCG GGT GCA GAG TGG GAA GGG CTG AGA GGA AGG CTG GAT AAG ACG ATG CTG

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3245 938

GAA AGA GAG GGT GGA GAA GGG GAT TTG AGG GGG GAG ACG ATG GTG CTG TGT GGG CCG AGG GGA
Glu Arg Glu Val Gly Glu Gly Asp Leu Arg Gly Gly Thr Met Val Leu Leu Cys Gly Pro Glu Gly

3311 960

ATG CAG AAT ATG GGT CAG GAG GTG TTG AAG GGG ATG GGG TGG AAG GAT GAG GAT GTA TTA GTT TTT
Met Gln Asn Met Val Arg Glu Val Leu Lys Gly Met Gly Trp Lys Asp Glu Asp Val Leu Val Phe

3377 982

TGA G CAAGTGTAA TTGCAAGGACC ATGCAATGAC ATGGAGGAC TACAGATGAC GITTGTGTG CACTGTCACA
End

3450

AGTGTGTA GCCTCCTCAC TCATCATGAA GCTGAGTGGG TGTTGTTGTG GTGGTGTTGGG GTGTTGGGG TGGTGTGGG

3530

GAAGTGCCT GGGAGAGACA TCTCCGCGGA GTGACAGCTG ACAAGCGTT

3580
Figure 10. The Direction of Transcription of the nit-3 Gene.
Northern blots of mRNA from nitrogen repressed and from nitrogen derepressed, nitrate induced mycelia were run in triplicate for detection with different probes. Lanes 1, 3 and 5 were loaded with nitrogen repressed mRNA while lanes 2, 4 and 6 contained nitrogen derepressed, nitrate induced samples. Lanes 1 and 2 were probed with the β-tubulin gene as an internal control. Lanes 3 and 4 were initially hybridized to one strand of nit-3 and lanes 5 and 6 were, likewise, probed with the other strand. Upon hybridization with the radiolabeled pBluescript vector, only one blot detects the nit-3 transcript and corresponds to the sense strand of nit-3.
Figure 11. S1 Nuclease Mapping of the Transcriptional Start and Stop Sites of \textit{nit-3}. Lanes R and DI represent the results from S1 nuclease mapping experiments using poly(A)$^+$ mRNA from repressed or induced cells, respectively. Lane P shows the intact untreated probe as a positive control used for mapping the 5'- and 3'-ends. The presence of the probe in the repressed lane for the 3'-end mapping experiment is presumed to have resulted from hybridization of the individual strands of the denatured probe with each other. Lane M is the 1 kb ladder (Bethesda Research Laboratories) which was used as the marker.
Figure 11
Figure 12. Primer Extension Analysis of the Transcriptional Start Sites.
Lanes 1 and 2 present primer extension studies with poly(A)$^+$ mRNA from induced and repressed cells, respectively. The exact positions of the sites as determined from reading a sequencing ladder which was run concomitently with extension reactions are shown.
Figure 12
Figure 13. Intron Splice Sites and Poly(A) Tail of the nit-3 Transcript.
Panel A shows the 15 nucleotide long poly(A) tract of the nit-3 cDNA, and panel B presents the intron splice sites that occur in the nit-3 gene. The single intron occurs within the sequence for the putative heme-binding domain of Neurospora nitrate reductase.
Figure 14. Sequence Comparison of the Single nit-3 Intron with the Introns of the Aspergillus niaD gene and with the Neurospora intron consensus sequence. Identical nucleotides are shown boxed and the percentage of perfect matches of each Aspergillus intron to the nit-3 intron is shown on the right. The first 6 and last 21 bases of the Aspergillus introns were compared to the first 6 and last 24 nucleotides of the Neurospora nit-3 intron. Distances between these two regions are shown as values within the intron sequences. Three nucleotides not considered for the comparison in the Neurospora intron sequence are in parentheses.
A. nidulans niaD

Intron I

Intron II

Intron III

Intron IV

Intron V

Intron VI

N. crassa

pit-3 intron

consensus seq

Figure 14
Figure 15. Amino Acid Comparison of the Deduced nit-3 Protein Sequence with Other Nitrate Reductases and Similar Redox Protein Sequences. Sequences were aligned by eye and open gaps are used as spacers to maximize homology. Identical matches are shown as ;, and the putative molybdenum-, heme- and flavin-binding domains are indicated as >m m<, >h h< and >f f<, respectively. Amino acid residues of particular interest and those referred to in the text are indicated with !, * and ↓. Numbering of the sequences is relative to Neurospora nitrate reductase and is shown on the right. Abbreviations: NCNR, Neurospora nitrate reductase (NR); ANNR, Aspergillus nidulans NR; ATNR, Arabidopsis thaliana NR; LENR, tomato NR; NSNR, tobacco NR; ZMNR, maize NR; CMNR, squash NR; BANR, barley NR; CRNR, Chlamydomonas reinhardtii NR; CLSO and RLSO, chicken and rat sulfite oxidases, respectively; HCb5, human liver cytochrome b5; Hb5R, human erythrocyte cytochrome b5 reductase.
Figure 15
Figure 16. Schematic diagram of the organization and putative sizes of the three cofactor-binding domains in *Neurospora* nitrate reductase. As a comparison, the *Aspergillus* nitrate reductase is also shown. MD, HEME and FAD identify the molybdenum, heme and flavin domains, respectively, and the squiggly lines between the domains reflect regions of little homology. Moreover, positions of the introns within two structural genes are also presented.
**N. crassa nit 3:**

```
ATG [△] TGA
Met 1  △  MD  △  HEME  △  FAD  △  Phe 982
NH₂  △  ~400-420 aa  △  ~70 aa  △  ~200 aa  △  COOH
```

**A. nidulans nia D:**

```
ATG [△] TGA
Met 1  △  I  △  II  △  III  △  IV  △  V  △  VI  △
NH₂  △  394 aa  △  66 aa  △  258 aa  △  Phe 873  △  COOH
```

Figure 16
Figure 17. Predicted Secondary Structural Motifs of the Deduced nit-3 Sequence. The secondary structure was predicted with the DNAStar (Madison, WI) and the IBI-Pustell softwares which use a combination of the Chou-Fasman (1978) and Garnier-Robson (1978) methods to determine structural patterns. Alpha-helices, β-sheets and turns are shown in bold, doubly underlined or overlined, respectively, for clarity. Boundaries of cofactor domains are identified as »m m«, »h h« and »f f« for the prosthetic groups, molybdenum, heme and flavin, respectively.
Figure 18. Sequence Analysis of Three nit-3 Mutant Alleles.
The nit-3 mutant alleles, V1M16, V1M4 and 14789A were cloned by PCR and sequenced. Panels A, B and C correspond to sequence comparisons of the mutants 14789A, V1M4 and V1M16, respectively, with the nit-3 wild-type nucleotide sequence. The V1M16 and 14789A mutant alleles were found to have a single base deletion while V1M4 contained a single point mutation. The mutated base and mutation are shown in parentheses; X, deletion of one base.
Figure 18
Figure 19. Schematic Localization of Three nit-3 Mutant Alleles in the nit-3 Gene. The position of each mutation is shown in reference to the cofactor-binding domains. The base position given is relative to the first transcriptional start site which is designated as +1. X represents the deletion of one base.
### MUTANT nit-3 ALLELES

<table>
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<th>MUTANT STRAIN</th>
<th>POSITION</th>
<th>MUTATION</th>
</tr>
</thead>
<tbody>
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<td>V1M4</td>
<td>822</td>
<td>TTA→TAA</td>
</tr>
<tr>
<td>14789A</td>
<td>2331</td>
<td>AAG→AXG</td>
</tr>
<tr>
<td>V1M16</td>
<td>2858</td>
<td>ATG→ATX</td>
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</table>
Figure 20. The \textit{pETNIT3ES} Expression Plasmid and the Expressed Protein Sequence. The upper panel depicts the \textit{pETNIT3ES} plasmid containing a 0.6 kb \textit{SmaII-BglII nit-3} cDNA fragment in the \textit{BamHI} site of the expression vector \textit{pET3c}. The T7 RNA polymerase promoter (T7P) was used to express a 28 kDa protein consisting of 204 amino acids of \textit{Neurospora} nitrate reductase heme domain that is flanked by 12 and 19 amino acids, respectively, at the amino and carboxyl termini (hatched boxes) derived from \textit{E. coli}. T\phi represents the position of the transcriptional termination sequence in the vector. The expressed protein sequence is shown in the lower panel with the \textit{E. coli} sequence on the first and last lines. The dot represents the first in-frame stop codon (UAA) in the vector sequence.
Figure 20
Figure 21. Expression of a Partial NIT3 Protein in *E. coli*.

A. Crude extracts from uninduced and induced *E. coli* cells containing *pETNIT3ES* were resolved in a 12% denaturing polyacrylamide gel and then stained with Coomassie Brilliant blue. Lane 1 was loaded with uninduced *E. coli* extract while lanes 2-4 consisted of bacterial extracts induced with IPTG for 1, 2 and 3 hours, respectively. After only 1 hour of induction, the most abundant protein about 28 kDa, corresponds to the calculated size for the *pETNIT3ES* expressed protein. The Rainbow protein markers (Amersham) were used to estimate size and are shown on the right in units of kDa.

B. Partial purification of the NIT3 protein was carried out by overnight electroelution of the expressed protein from a polyacrylamide gel. An aliquot of the electroeluted protein was run in a 12% SDS-polyacrylamide gel and stained as before to determine the extent of purification. After one round of electroelution, the expressed protein is sufficiently pure for polyclonal antibody production. Lane M, Rainbow marker; lanes 1 and 2, two different preparations of partially purified, *E. coli* expressed NIT3 protein.

C. The specificity of the anti-NIT3 polyclonal antibody was verified by Western blot analysis of extracts from uninduced and induced *E. coli* containing either the *pETNIT3ES* plasmid or the *pET3c* expression vector, as a negative control. One of two duplicate gels was stained with Coomassie Brilliant blue dye and is shown to the left; the gel to the right was probed with the anti-NIT3 antibody. The serum was diluted 1 X 10^4 in TBST buffer and 10 mL of buffer was used for hybridization. The primary antibody was not preabsorbed with *E. coli* extract and accounts for the detection of other nonspecific bands. However, specificity of the anti-NIT3 antibody was clearly observed for the NIT3 expressed protein as shown in lane 4. Lanes 1 and 2 are, respectively, uninduced and induced extracts containing *pET3c*, while lane 3 is uninduced extract with the *pETNIT3ES* plasmid.
Figure 21
Figure 21 (continued)

C.
Figure 22. Western Blot Analysis of Three Nitrate Reductase Mutant Proteins. Crude extracts of nitrogen repressed and nitrogen derepressed, nitrate induced mycelia from three nit-3 mutant strains 14789A (lanes 3 & 4), V1M16 (lanes 5 & 6) and V1M4 (lanes 7 & 8) as well as from wild-type 74A (lanes 1 & 2) were electrophoresed in a 7.5% denaturing polyacrylamide gel. The proteins were electroblotted onto two layers of nitrocellulose filters and probed with the anti-NIT3 antibody. The polyclonal antibody was diluted 1:1000 in TBST buffer and preabsorbed with nitrogen repressed Neurospora wild-type extract to increase its specificity. Detection of the proteins was by chemiluminescence using the horseradish peroxidase-linked secondary antibody as described in Experimental Procedures. The nitrate reductase subunit sizes for V1M16 and 14789A were estimated to be 100 and 85 kDa in comparison to the wild-type monomer size and with the protein Rainbow markers (lane M). The extra smaller bands observed with the V1M16 samples are postulated to have resulted from partial protein degradation.
1 = repressed 74A
2 = derep-induced 74A
3 = repressed 14789
4 = derep-induced 14789
5 = repressed V1M16
6 = derep-induced V1M16
7 = repressed V1M4
8 = derep-induced V1M4

Figure 22
Figure 23. Transformation Assays of *nit-3* Nested Deletion Subclones with the *nit-3* Mutant Strain 14789A. A set of nested deletion subclones was transformed into the *nit-3* mutant strain 14789A to determine if targeting was occurring with the *nit-3* gene. A restriction map of the *nit-3* gene and relative positions of the cofactor-binding sites are shown at the top. The three asterisks represent NIT-2 binding sites as determined from previous footprinting experiments (Fu and Marzluf 1990a). The deletion plasmids are shown aligned with physical map. The single mutation in 14789A is shown by the dot in the FAD domain. Plus (+) and minus (-) signs to the right represent the presence or absence of transformants with the corresponding deletion subclone. In all transformation assays, 1 μg of DNA was used.
Figure 23
Figure 24. Nitrate Reductase Assays of Selected nit-3 Deletion Transformants.

Nitrate reductase assays were carried out in duplicate on three independent colonies for each of the deletion transformants \textit{pN3RVK}, \textit{pN3PA33}, \textit{pN3R205} and \textit{pN3A317}. All samples were grown under nitrogen repressed (R) and nitrogen derepressed, nitrate induced (DI) conditions. The specific activities, which is defined as nmoles of nitrite produced per minute per microgram of protein, of the transformants were compared to that of wild-type 74A. Standard deviations for each are shown above the bars.
Figure 24

Specific Activity (U/ug)

Nit-3 Constructions

74A RVK A33 A205 A317

R DI R DI R DI R DI R DI R DI
Genomic DNA was isolated from wild-type strain 74A and from the deletion transformants *pN3RVK*, *pN3PA33*, *pN3RA205* and *pN3BA317*. Two independent transformed colonies were analyzed for each deletion transformant with the exception of *pN3RA205* for which only one was examined. The arrow indicates the position of the *EcoRI* fragment for the wild-type *nit-3* gene (lane 1). The lanes are designated as follows: lanes 2 and 3 contain samples from *pN3RVK*, lanes 4 and 5 of *pN3PA33*, lane 6 of *pN3RA205* and lanes 7 and 8 of *pN3BA317*. 
Figure 26. Western Analysis of the Nitrate Reductase Protein Expressed in the Deletion Transformants. Nitrogen derepressed, nitrate induced mycelia were used to obtain crude cellular extracts from wild-type strain 744 (lane 1), mutant strain 14789A (lane 2) and deletion transformants \( pN3RVK, \ pN3P\Delta33, \ pN3R\Delta205 \) and \( pN3\Delta317 \) (lanes 3-6, respectively). The protein extracts were resolved in a 7.5% denaturing polyacrylamide gel and probed with the anti-NIT3 antibody. The secondary antibody used for detection was linked to streptavidin-alkaline phosphatase which accounts for the cross-reacting band observed in all of the lanes. The large arrow points to the position of wild-type nitrate reductase protein whereas the smaller arrow shows the location of the mutant 14789A NIT3 protein. The presence of the mutant 14789A protein in the deletion transformants is due to the use of heterocaryons for analysis.
Table 1. Genotypic Characterization of F1 Progeny

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<td>nd</td>
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<td>RIP70a</td>
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Wild-type spheroplasts were transformed with an XbaI-KpnI clone of *nit-3* as described in the Results. Homocaryons possessing multiple copies were crossed with wild-type strain 74, and the putative *nit-3* RIP mutants were identified by their failure to grow on nitrate. These putative *nit-3* mutants were then crossed with wild-type and with the conventional *nit-3* mutant strain (14789). The progeny were tested for growth on nitrate, and the values in the table represent the number of nitrate non-utilizing isolates. Abbreviation: nd, not determined.
Table 2. Genotypic Characterization of the F2 Progeny

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The F1 parent strains were crossed with wild-type strain 74a and three of the nitrate non-utilizing progeny were testcrossed with wild-type and with the *nit-3* conventional mutant 14789a. The subsequent progeny were scored for their ability to utilize nitrate. The notation, nd, stands for not determined. Values in the table show the fraction of progeny which were nitrate non-utilizers.
Figure 27. Analysis of nit-3 RIP Mutants by Southern Blot Experiments. Genomic DNA from F1 progeny, RIP11a and RIP17A (in lanes 2 and 3) as well as from the F2 progeny, RIP44A, RIP53a and RIP57A (in lanes 4, 5 and 6, respectively), were digested with PstI and probed with the wild-type nit-3 gene. Lane 1 shows the restriction pattern for wild-type nit-3 with PstI. For all of the mutants samples except RIP44A, the wild-type PstI fragment is missing, due to the mutational loss of one or more PstI restriction sites.
Figure 28. Southern Analysis of Selected RIP Progeny.
Genomic DNA from RIP progeny of both F1 and F2 generations were digested with EcoRI and probed with a 1.5 kb HindIII-BglII nit-3 fragment that detected a 3.8 kb EcoRI fragment in wild-type genomic nit-3 (lane W). Lanes A, B and C, F1 progeny, RIP17A, RIP70a and RIP54a, respectively, with their F2 progeny adjacent to them. Lanes 1, 2 and 3, F2 progeny RIP9/4, RIP22/4 and RIP24/4 from a RIP17A x WT cross; lanes 4 and 5, F2 progeny RIP53a and RIP57/1, and lane 6 the F2 progeny RIP444 which were produced by crossing RIP70a and RIP54a, respectively, with the wild-type strain. The condition for washing the filter was in 0.1X SSC, 0.1% SDS twice for 30 minutes at 50°C. This condition, which is much less stringent than for most Southern blots was used due to the decrease hybridization of some of the F2 progeny (in particular, RIP22A and RIP24A) with the nit-3 wild-type probe. The numbers on the right were from size markers, using the 1 kb ladder (BRL).
Table 3. Nitrate reductase and partial enzyme activity assays for wild-type and various nit-3 mutant strains measured under non-inducing (U) and inducing (I) conditions. For the FAD partial activity assays, the low basal level measured in the nit-3 mutants is presumed to be due to the presence of nitrite reductase, which also uses FAD. All assays were performed in duplicate and specific activity is defined as nmoles of nitrite produced per minute per milligram of protein. Abbreviations: NADPH-NaR, nitrate reductase assay; MO-HEME, methyl viologen-nitrate reductase assay; FAD, ferricyanide-nitrate reductase assay; nd, not determined.

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</table>
Figure 29. Partial Sequence of the 5'–Region of the *nit-3* Mutant Allele RIP22A. A 1.1 kb fragment was cloned by PCR and sequenced. The sequenced fragment was compared with the corresponding region of the *nit-3* wild-type nucleotide sequence, shown on the left. Point mutations are shown in bold.
Figure 29
Figure 30. Partial Sequence of the 3'-Region of the *nit-3* Mutant Allele RIP22A. A 1 kb RIP22A fragment was cloned by PCR and the resulting sequence was compared with the corresponding region of the wild-type *nit-3* sequence as shown on the left. A new *EcoRI* restriction site, as suggested by Southern blot experiments, was found to occur within this fragment of the mutant and is shown in bold.
Figure 30
Figure 31. Sequence Analysis of the Newly Generated nit-3 Mutant Allele RIP224 at Its 5'-end. An abbreviated restriction map of nit-3 is shown at the top, and the positions of the PCR primers used to amplify the region analyzed are indicated. The nucleotide and corresponding amino acid sequences are given for a 790 nucleotide region in the 5'-region that begins with the ATG translational start codon. All G and C residues which have been mutated by the RIP process are shown underlined. Amino acid residues that have been altered as a result of the induced point mutations are shown directly under the wild-type one. Nonsense mutations are depicted as Stop.
**Figure 31**
Figure 32. Sequence Analysis of the New nit-3 Mutant Allele RIP22A in the 3'-Region. The DNA and amino acid sequences are provided for a 206 base pair region in the 3'-region of the nit-3 gene. The guanine and cytosine bases of the wild-type sequence which have undergone transition mutations (G to A and C to T) are underlined, and the amino acid substitutions that occurred as a result of RIP are shown directly under the original residue. The single nonsense mutation is denoted as Stop, and the 61 nucleotide intron is shown in lower case. The abbreviated restriction map of nit-3 with the locations of the PCR primers used to amplify the region analyzed is shown at the top.
Figure 32
Figure 33. Western Analysis of the RIP Mutant Nitrate Reductase Protein. 
Crude extracts from nitrogen repressed and nitrogen derepressed, nitrate induced mycelia were obtained from wild-type 74A and nit-3 mutant allele RIP22A and analyzed by Western blot experiments. Lanes 1 and 2 are wild-type samples grown under repressed and induced conditions, respectively, while lanes 3 and 4 are repressed and induced RIP22A extracts, respectively. The polyclonal anti-NIT3 antibody detected the nitrate reductase subunit only in the lane containing the induced wild-type extract. As expected, no nitrate reductase was present in the mutant strain RIP22A. M, size marker lane with the 200 kDa size indicated.
Figure 33
Figure 34. Transformation Assays of the RIP22A Mutant Strain with Nested Deletion Subclones. The set of nested deletion subclones, which had previously been used to demonstrate targeting in the nit-3 mutant strain 14789A (Figure 23), were transformed into RIP22A spheroplasts by the standard transformation procedure. The restriction map of nit-3 and relative positions of the cofactor-binding domains are shown at the top. Asterisks represent the three NIT2 binding sites in the nit-3 promoter. The extent of the lines represents the sizes of the corresponding subclones. Plus (+) and minus(-) signs were used to illustrate positive or negative transformation results.
Figure 34

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<tr>
<td>pN3Δ620</td>
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<tr>
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<td>+</td>
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** RIP22
Figure 35. Nitrate Reductase Assays of Transformants Containing pN3Δ3UT. Nitrate reductase assays were performed on RIP22A and 14789A spheroplasts transformed with the plasmid, pN3Δ3UT, which has the entire 3'-nontranslated region of nit-3 missing. Specific activity is defined as nmoles of nitrite produced per minute per microgram of protein.
Figure 36. The Tertiary Structure of Bovine Liver Cytochrome b₅.
The crystal structure of calf liver cytochrome b₅ was determined by Mathews et al. (1972), and is presented in the upper panel. The heme-binding crevice is shown with the heme porphyrin structure at the top of the molecule. The numbering of the figure corresponds to the bovine liver cytochrome b₅ amino acid sequence (BCb₅), which is shown in alignment with that of human cytochrome b₅ (HCb₅) and of the heme domain in Neurospora nitrate reductase (NCNR; lower panel). The darkened circles in the tertiary structure and the asterisks in the sequence represent invariant residues. The amino acids that were mutated and analyzed in this study are labeled in the upper panel and underlined in the lower panel.
Figure 36
Table 4. Transformation Frequency of \textit{in vitro} Mutagenized \textit{nit-3} Genes with the Host Strain RIP22A. Each transformation was performed in duplicate with 1 μg of transforming DNA. The numbers following each amino acid residue corresponds to their position in the primary sequence for \textit{Neurospora} nitrate reductase.

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<tr>
<th>Name</th>
<th>Mutation</th>
<th>Transformation Frequency</th>
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<tbody>
<tr>
<td>W</td>
<td>None (\textit{nit-3}⁺)</td>
<td>273 312</td>
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<tr>
<td>H652A</td>
<td>His-652 to Ala</td>
<td>83 104</td>
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<tr>
<td>F671A</td>
<td>Phe-671 to Ala</td>
<td>18 13</td>
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<tr>
<td>W635F</td>
<td>Glu-633 to Ala</td>
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<tr>
<td>E633A</td>
<td>Trp-635 to Phe</td>
<td>283 187</td>
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<tr>
<td>H675A</td>
<td>His-675 to Ala</td>
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<tr>
<td>I659G</td>
<td>Ile-659 to Gly</td>
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<tr>
<td>S694A</td>
<td>Ser-694 to Ala</td>
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<tr>
<td>S676A</td>
<td>Ser-676 to Ala</td>
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</table>
Figure 37. Southern Blot Analysis of Heme Mutants H652A, F671A and W635F. Genomic DNA from homocaryons of heme mutants H652A, F671A and W635F along with that of nit-3+ transformant (W) was isolated and analyzed by Southern blot experiments. Lane H presents the EcoRI digestion pattern for the host strain RIP22A (control) whereas lanes 1-3, lanes 4-7, lanes 8-11 and lanes 12-15 show the restriction digests of W, H652A, F671A and W635F, respectively. For each mutant and wild-type, at least three independent homocaryons were analyzed. Homocaryons which had a single copy of the transformed nit-3 genes were used for further studies.
Figure 38. Southern Blot Analysis of the Heme Mutant E633A.
Southern blot experiments were performed as described in Figure 38. Lane H contains genomic DNA of the host strain digested with EcoRI. Lanes 1-4 show the restriction digestion pattern of four individual homocaryons. Those containing single copies of the mutagenized nit-3 gene were further examined.
Figure 39. Southern Blot Analysis of the Heme Mutants H675A, I659G, S694A and S676A. Southern analysis was carried out as described in the legend for Figure 38. Lanes 1-10 contain genomic DNA from individual homocaryons of heme mutant H675A while lanes 11-19, 20-25 and 26-33 are for heme mutants I659G, S694A and S676A, respectively. Since heme mutants H675A, I659G and S676A could not be selected on restrictive medium, a larger population of homocaryons were analyzed. Lane H represents EcoRI-digested genomic DNA from the host strain RIP22A which was run as a control.
Figure 39 (continued)
Table 5. Nitrate Reductase and Associated Enzyme Activity Assays for wild-type strain 74A (74A), wild-type nit-3 transformant (W) and the heme mutants. Specific activities for the NADPH-NaR and MVH-NaR assays are defined as nanomoles/minute/mg protein, while those for the CCR-NaR and Fe-NaR have units of micromoles/min/mg. The levels of activity for the NADPH-NaR and MVH-NaR assays are relative to the activity of the nit-3 wild-type transformant (W) under induced conditions. Abbreviations: NADPH-NaR, nitrate reductase assay; MVH-NaR, molybdenum and heme domains assay; CCR-NaR, assay for the FAD and heme domains; Fe-NaR, assay for the flavin domain; U, repressed condition; I, induced condition; nd, not determined.

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<td>15.1</td>
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Figure 40. Relative Nitrate Reductase Levels in the Heme Mutants.
Nitrate reductase activity was compared to that of the wild-type \textit{nit-3} transformant (W) for all of the heme mutants. The average specific activity for the wild-type transformant was $3.1 \pm 0.2$ U/mg. The induced activities are shown as solid bars while the stippled boxes represent activity under nitrogen repressed conditions. At least three individual homocaryons were assayed for each mutant and the averages of their activity was used to calculate the corresponding relative specific activities. Abbreviations: H1, H652A; H2, F671A; H3, W635F; H4, E633A; H5, H675A; H6, I659G; H7, S694A; H8, S676A.
Figure 40
Figure 41. Relative Methyl Viologen-Nitrate Reductase Activity Levels in the Heme Mutants. The ability to utilize reduced methyl viologen as an electron donor for nitrate reduction was measured for each heme mutant and compared to that of the wild-type nit-3 transformant (W). The average specific activity for the wild-type transformant was 1.7 ± 0.2 U/mg. Three individual homocaryons were assayed for each mutant and the averages of their activities were used to calculate the corresponding relative specific activities. Abbreviations: H1, H652A; H2, F671A; H3, W635F; H4, E633A; H5, H675A; H6, I659G; H7, S694A; H8, S676A.
Figure 41
Figure 42. Stability of the Nitrate Reductase Protein in the Heme Mutants.
Nitrogen repressed and nitrogen derepressed, nitrate induced protein extracts were prepared from a representative homocaryon for each of the heme mutants and probed with the anti-NIT3 antibody. Detection was with the horseradish peroxidase-linked secondary antibody using chemiluminescent substrates. In all of the Western blots, lane 74A presents the wild-type nitrate reductase protein under induced conditions and was dually used as a size marker and control. To facilitate comparisons, nitrogen repressed and nitrate induced samples were loaded next to each other for each heme mutant. Lane M is the marker lane and the 200 kDa protein is indicated. Panel A: Western blot of repressed and induced heme mutants F671A (lanes 1 and 2, respectively) and H675A (lanes 3 and 4, respectively). Lane 1 in both panels B and C contains the protein extract from the nitrate induced RIP22A host strain and was used as a negative control. Panel B: Western analysis of heme mutants W635F (lanes 2 and 3) and S676A (lanes 4 and 5). Panel C: heme mutants E633A (lanes 2 and 3) and I659G (lanes 4 and 5). Panel D: lanes 1 and 2 are Western analyses of the wild-type 74A under repressed and induced conditions, respectively, while lane 3 shows the mutant nitrate reductase protein of induced 14789A which was used as a size marker. Lane 4 is the protein extract of induced RIP22A, and lanes 5 and 6, the heme mutant H652A. Panel E: lanes 1 and 2 of panel E are samples from induced wild-type strain 74A and the nit-3 mutant host strain. Lanes 3 and 5 are nitrogen-repressed samples of heme mutant S694A and the nit-3 wild-type transformant, respectively; lanes 4 and 6 are the extracts from the same induced strains.
Figure 42
Figure 42 (continued)

D.  

E.  

200kDa

200kDa
Figure 43. Heme-binding Studies on the Mutants H652A and H675A.
The two histidine mutants, H652A and H675A were examined for their content of heme.
Protein extracts were prepared from repressed and induced mycelia of wild-type strain
74A (lanes 1 and 2, respectively) and heme mutants, H652A (lanes 3 and 4, respectively)
and H675A, (lanes 5 and 6, respectively). Resolution was in a 7% nondenaturing
polyacrylamide gel. The arrow indicates the position of the nitrate reductase protein
which is stained for heme with 0.2% benzidine-hydrogen peroxide (Panel A). Panel B
is a duplicate gel stained with Coomassie Brilliant blue to show that equal amounts of
protein extract had been loaded per lane. In both gels, the β-galactosidase protein was
used as a size marker and its position is shown to the left.
Figure 43
Figure 44. Western Analysis of nit-3 Amber Mutant and Suppressed Mutant Strains. Crude extracts of four nit-3 amber mutant strains (KGP1222, KGP026, KGP0213 and KGP1211) and their corresponding suppressor strains (prefixed with SSU-1 in the figure) were prepared from repressed (R) and induced (DI) mycelia and analyzed by Western blot experiments. Nitrogen derepressed, nitrate induced (DI) samples of wild-type strain 74A and the conventional nit-3 mutant strain 14789A were used as controls and as size markers. Lane M represents the protein marker lane with the position of the 200 kDa protein indicated to the left.
Figure 44
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Lee H, Fu Y, Marzluf GA. (1990b) Nucleotide sequence and DNA recognition elements of alc, the structural gene which encodes allantoicase, a purine catabolic enzyme of *Neurospora crassa*. Biochem 29:8779-8787.


