Analysis of Wild-Type and Mutant Plant Nitrate Reductase Expressed in the Methylo trophic Yeast Pichia pastoris

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Recombinant Arabidopsis thaliana NADH:nitrate reductase (NR; EC 1.6.6.1) was produced in the methylo trophic yeast Pichia pastoris and purified to near-electrophoretic homogeneity. Purified enzyme had the spectral and kinetic properties typical of highly purified NR from natural plant sources. Site-directed mutagenesis altering several key residues and regions was carried out, and the mutant enzyme forms were expressed in P. pastoris. When the invariant cysteine residue, cysteine-191, in the molybdo-pterin region of the A. thaliana NIA2 protein was replaced with serine or alanine, the NR protein was still produced but was inactive, showing that this residue is essential for enzyme activity. Deletions or substitutions of the conserved N terminus of NR retained activity and the ability to be inactivated in vitro when incubated with ATP. Enzyme with a histidine sequence appended to the N terminus was still active and was easily purified using metal-chelate affinity chromatography. These results demonstrate that P. pastoris is a useful and reliable system for producing recombinant holo-NR from plants.

Assimilatory NR (EC 1.6.6.1–3) catalyzes the reduction of nitrate to nitrite, the first committed step in the nitrate assimilation pathway (Solomonson and Barber, 1990; Hoff et al., 1994; Crawford, 1995). NR is a large, metalloflavoenzyme that exists as a homodimer with a monomer mass of approximately 100 kD. Each subunit consists of three functional regions or domains, with each region associated with a redox center or cofactor (FAD, heme-Fe, and MoCo) that transfers electrons from NAD(P)H to nitrate (Fig. 1; Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Crawford, 1995, 1996). Electron flow is from NAD(P)H to FAD to heme-Fe to MoCo to nitrate (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Crawford, 1995, 1996). Artificial electron donors (such as methyl viologen or bromphenol blue) or acceptors (such as Cyt c) can be used to assay several nonphysiological partial activities of NR (Solomonson and Barber, 1990). Extensive amino acid sequence similarities exist between each of the three functional regions of NR and other redox enzymes and proteins (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Hoff et al., 1994; Crawford, 1995). The MoCo-binding region of NR is similar to the MoCo-binding region of sulfite oxidase; the heme-Fe-binding domain is similar to proteins in the Cyt b5 superfamily and the Cyt b reductase region, which contains the FAD-binding domain, is most similar to NADH:Cyt b5 reductase (Calza et al., 1987; Crawford et al., 1988; Campbell, 1996). The Cyt b reductase fragment of NR has been shown by structural analysis to be a member of the Fd NADP+ reductase family of flavoenzymes (Lu et al., 1994; Campbell, 1996).

Although the biochemistry of NR has been studied for more than four decades, the complete three-dimensional structure is not known, and mechanistic details of enzyme catalysis are lacking because of the limited quantities of pure NR that are available (Campbell, 1996). Until recently, the study of mutant NR forms has been restricted to the analysis of a few plant mutants (Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Hoff et al., 1994). One approach to overcome these limitations is to express NR in a heterologous system. Toward this end, various fragments of NR have been expressed in Escherichia coli, including the Cyt b reductase, the heme-binding domain, and the combination of these two, known as the Cyt c reductase fragment (Hyde and Campbell, 1990; Campbell, 1991; Cannons et al., 1993; Dwivedi et al., 1994; Gonzalez et al., 1995; Quinn et al., 1996). The Cyt c reductase fragment of spinach (Spinacia oleracea L.) NR has also been produced in the methylo trophic yeast Pichia pastoris at much higher levels than could be attained in E. coli (Shiraishi and Campbell, 1997). Biochemical characterization and site-directed mutagenesis were performed on these recombinant proteins. The Cyt b reductase fragment of corn NR has been crystalized, its three-dimensional structure determined, and the mechanism of electron transfer analyzed (Lu et al., 1995; Ratnam et al., 1995; Campbell, 1996). Expression of the MoCo-binding fragment of NR alone has not been reported, but sulfite oxidase, which shows striking similarity to the MoCo and heme domains of NR, has been expressed in E. coli (Garrett and Rajagopalan, 1994, 1996). Crystal structures of other MoCo enzymes such as DMSO reductase have been described (Schindelin et al., 1996), but these enzymes have no sequence similarity to NR or sulfite oxidase.

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An important feature of NR is that it is regulated by a posttranslational mechanism that rapidly and reversibly inhibits the enzyme in response to several signals, including light/dark transitions (Kaiser and Huber, 1994). This mechanism involves phosphorylation of a Ser in the hinge 1 region of NR (Ser-547 in spinach and Ser-534 in Arabidopsis) and interaction with an inactivator protein (14–3-3 protein) in the presence of magnesium (Douglas et al., 1995; Bachmann et al., 1996a, 1996b; Huber et al., 1996; Moorhead et al., 1996; Su et al., 1996). NR is reactivated by dephosphorylation by type 2A protein phosphatase (Mackintosh, 1992). Phosphopeptide analysis of NR has revealed that multiple Ser residues are phosphorylated in NR (Huber et al., 1992; Labrie and Crawford, 1994), but only the phosphorylation of Ser-543 in spinach correlates with the loss of NR activity (Huber et al., 1992; Douglas et al., 1995; Bachmann et al., 1996b). The production of recombinant fragments of spinach NR as targets for the spinach NR protein kinase was instrumental in leading to the identification of hinge 1 as a regulatory region of NR (Bachmann et al., 1996b).

Two recent findings have opened up new avenues for the efficient site-directed mutagenesis and structural/functional characterization of holo-NR. In fungi, mutant forms of the species Aspergillus NADPH:NOR have been expressed in an niaD deletion mutant (Garde et al., 1995). In this study a Cys (Cys-150) in the MoCo-binding region and a His (His-547) in the heme-Fe domain were identified as essential residues for fungal NR activity. Cys-150 (equivalent to Cys-191 in Arabidopsis NR) corresponds to an invariant Cys residue among all NR and sulfite oxidase sequences, which is part of the signature sequence CAGNRR for this group of enzymes (Barber and Neame, 1990; Garrett and Rajagopal, 1994), and has been shown to be a ligand to the molybdenum in rat liver sulfite oxidase (Garrett and Rajagopal, 1996). His-547 is thought to provide an axial ligand to the heme-Fe domain (Meyer et al., 1991; Garde et al., 1995). For plants, a functional NADH:NOR has been expressed in P. pastoris (Su et al., 1996). Arabidopsis Nia2 NR was expressed under the control of the P. pastoris alcohol oxidase 1 promoter as previously described (Su et al., 1996). P. pastoris was initially grown in 500-mL cultures containing YPD media (1% yeast extract, 2% peptone, and 2% Glc, all w/v) to an A600 of 10. The log-phase cells were washed and then resuspended to an A600 of 5 in 1 L of modified MM media (2.8% [w/v] yeast nitrogen base with ammonium sulfate, 100 mM potassium phosphate, pH 6.0, 4 x 10^{-5}% biotine, 0.2 mM sodium molybdate, and 1% methanol [v/v]) to induce NR expression. Methanol (1% [v/v]) and sodium molybdate (0.2 mM) were added to the culture every 24 h. After a 72-h induction period the cells were collected by centrifugation, washed, and then resuspended in extraction buffer containing 50 mM sodium phosphate, pH 7.3, 1 mM EDTA, and 5% (v/v) glycerol to an A600 of 50 to 100. The cells were broken with a Bead Beater (BioSpect Products, Bartlesville, OK) using 0.5-μm glass beads by processing for 5 min using 30-s bursts followed by 1-min cooling periods. The crude extract was then centrifuged at 15,000g for 20 min at 4°C.

NR assays of crude extracts and purified samples were performed as previously described (Campbell and Smarrelli, 1978; Redinbaugh and Campbell, 1985). One unit of NR activity is defined as 1 μmol of nitrate produced/min. For the purification of wild-type NR, crude cell extracts were subjected to a 30 to 45% ammonium sulfate precipitation. The resulting pellet was dissolved in 50 mM sodium phosphate, pH 7.3, and 1 mM EDTA (buffer A) and batch-bound to Blue-Sepharose (Campbell and Smarrelli, 1978). The Blue-Sepharose with bound NR was collected by vacuum filtration, thoroughly washed with buffer A, and packed in a 1.5-cm column. The column was eluted with 0.1 mM NADH in buffer A. Fractions containing NR activity were pooled, buffer exchanged, and concentrated with 25 mM Mops (ultrapure grade, Calbiochem), pH 7.0, using a Centriprep 30 concentrator (Amicon, Beverly, MA). Next, the partially purified sample was applied to a 1.5-M column of 5’-AMP Sepharose (Pharmacia). The column was washed with 5 column volumes of 50 mM Mops, pH 7.0, and 1 mM EDTA (buffer B). NR was then eluted with 0.1 mM NADH in buffer B. Fractions containing more than 2 units/mL of NR activity were pooled and concentrated, with the buffer exchanged to 25 mM Mops, pH 7.0.

NR Kinetics and Spectra

NR samples purified as described above were used. Kinetic experiments were performed at 25°C in 1 mL of buffer more, we showed that the N-terminal amino acids (residues 1-11) are not required for the ATP-dependent inhibition of NR and can be replaced with a hexahistidine sequence, which facilitates purification of NR for in vitro regulatory studies.

MATERIALS AND METHODS

Recombinant Arabidopsis NADH:NR Production and Purification

NR was expressed in the methylo trophic yeast Pichia pastoris strain GS115 from the Arabidopsis NIA2 NR cDNA pAtc-46 (Crawford et al., 1988) under the control of the P. pastoris alcohol oxidase 1 promoter as previously described (Su et al., 1996). P. pastoris was initially grown in 500-mL cultures containing YPD media (1% yeast extract, 2% peptone, and 2% Glc, all w/v) to an A600 of 10. The log-phase cells were washed and then resuspended to an A600 of 5 in 1 L of modified MM media (2.8% [w/v] yeast nitrogen base with ammonium sulfate, 100 mM potassium phosphate, pH 6.0, 4 x 10^{-5}% biotine, 0.2 mM sodium molybdate, and 1% methanol [v/v]) to induce NR expression. Methanol (1% [v/v]) and sodium molybdate (0.2 mM) were added to the culture every 24 h. After a 72-h induction period the cells were collected by centrifugation, washed, and then resuspended in extraction buffer containing 50 mM sodium phosphate, pH 7.3, 1 mM EDTA, and 5% (v/v) glycerol to an A600 of 50 to 100. The cells were broken with a Bead Beater (BioSpect Products, Bartlesville, OK) using 0.5-μm glass beads by processing for 5 min using 30-s bursts followed by 1-min cooling periods. The crude extract was then centrifuged at 15,000g for 20 min at 4°C.

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containing 50 mM Mops, pH 7.0, by following the decrease in A$_{440}$ using a 1-cm cuvette and a UV/Vis spectrophotometer (model no. 1201, Shimadzu, Kyoto, Japan). The concentration of the substrates varied from 0.5 to 20 μM for NADH (Sigma) and 2.5 to 60 μM for potassium nitrate. The results of three initial velocity experiments were analyzed using Enzpack software (Elsevier Biosoft, Cambridge, UK). Spectra of oxidized and NADH-reduced samples were determined at 25°C using a UV/Vis diode array spectrophotometer (model no. 8453, Hewlett-Packard).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed as previously described (Higuchi, 1990) using the Arabidopsis cDNA clone pAtc-46 (Crawford et al., 1988). For the point mutations, a 1.8-kb SmaI fragment from pAtc-46 was subcloned into the Bluescript (KS-) vector (Stratagene) to generate a template for PCR. In the first round of PCR T7 and T3 primers were used as outside primers, whereas inside primers were oligonucleotides with designed mutations. One strand of the oligonucleotides for each mutation is shown; the reverse-strand primers were complementary: S216A, 5'-TCCGCCGGAGTTGCAICTCCTGCTGTG-3'; S261A, 5'-GGAACCTGTGTCGCAATAACG-GAAGCC-3'; S266A, 5'-AAATACGGAACGCAATCAA-GAAGGAA-3'; S324A, 5'-ACAACCTAAGAGAAGCGCGG-GAATTCTTAC-3'; S365A, 5'-CTAAACATATAACGCCGTGTGATTACGACG-3'; S438A, 5'-TGGTGTTTTTGGCACTTGAAGGGTTAGGAG-3'; S191S, 5'-GCGACGCTAGTCAGCGGGGGGAACCGC-3'.

The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'. The final PCR products were gel-purified and then digested with AcI and XhoI to produce a 1-kb (112-1098) fragment that was used as primers: 5'-ACACTTTCCAACATGGTAA-3' and 5'-GCGATTATCTACCATGTTGGAAAG-3'.

Figure 1. The diagram shows the location of the three cofactor-binding regions or domains, the N-terminal leader, and the hinge regions along the linear sequence of NR. Amino acids that were mutated in this study are shown along with the phosphorylated Ser-534. wt, Wild type.

Expression of Mutant NR Proteins

Plasmids containing the desired mutations were transformed into P. pastoris strains GS115 and SMD1168 by electroporation as previously described (Becker and Guar- ente, 1990; Su et al., 1996). Growth and preparation of protein extracts from the transformants were as described previously, except no Triton X-100 was used in the extraction buffer (Su et al., 1996). For each mutant, at least 10 transformants were initially screened for NR expression, and the transformants showing the highest level of NR activity were used for further characterization. The NR assay condition for the mutant NRs was as described previously (Su et al., 1996). The partial purification of NR with a His-tag at the N terminus was performed as follows. Frozen P. pastoris cells (10 g) were suspended in 10 mL of 50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 5% glycerol (v/v), and 1 mM PMSF. The suspended cells were lysed with a French press. The crude cell lysate was centrifuged at 16,000 rpm for 10 min. The supernatant was incubated with 5 mL of 80% (v/v) CL-6B Sepharose (Pharmacia) at 4°C for 30 min and then centrifuged for 5 min at 12,000 rpm. The supernatant was then incubated at 4°C for 30 min with 2 mL of a 50% slurry of nickel-nitrilotriacetate gel agarose (Qiagen, Chats- worth, CA) in nickel-nitrilotriacetate gel buffer (50 mM sodium phosphate, pH 7.5, 20 mM imidazole, 10 mM 2-mercaptoethanol, and 5% glycerol [v/v]) and then centrifuged at 4000 rpm for 30 s. The column resin was washed with 20 mL of 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 5% (v/v) glycerol, 300 mM NaCl, and 10 mM mercaptoethanol, resuspended in 5 mL of Mops buffer (50 mM Mops, pH 7.5, 1 mM EDTA, and 5% [v/v] glycerol), and...
then transferred to a 10-mL column. The column was washed with 5 mL of Mops buffer, and then NR was eluted with 4 mL of Mops buffer with 200 mM imidazole. Column fractions with maximal NR activity were dialedyzed in Mops buffer and stored at −80°C. Overall recovery was 50% of the starting material with a final NR specific activity of 4 units/mg (180 μg of protein).

**In Vitro Inhibition Assay and Immunoblot Analysis**

The inhibition assay was performed as previously described (Su et al., 1996). Briefly, *P. pastoris* extracts containing NR (0.001–0.003 unit) were mixed with 150 μL (approximately 300 μg of protein) of desalted protein extracts from G4–3, a NR double mutant of Arabidopsis (Wilkinson and Crawford, 1993). The mixture was incubated at 22°C for 20 min in the presence or absence of 5 mM ATP, and the extent of inhibition was determined with an NR assay.

The immunoblotting procedure was as previously described (Su et al., 1996), except that an Immobilon-P membrane (Millipore), instead of nitrocellulose, and a Protean II gel apparatus (Bio-Rad) were used. Anti-NR antibodies prepared against Arabidopsis NIA2 NR purified from *Escherichia coli* (Wilkinson and Crawford, 1991) were used. Bound antibody was visualized by secondary antibodies and horseradish peroxidase. For the native polyacrylamide gel analysis, *P. pastoris* was lysed in extraction buffer (50 mM Mops, pH 7.5, 1 mM EDTA, and 5% [v/v] glycerol with 0.4 μg/mL leupeptin and 1 μg/mL pepstatin). Tris-HCl (pH 6.8) and glycerol were added to the samples to a final concentration of 20% and 10%, respectively. The samples were loaded onto a 5 to 20% gradient of Ready Gels for the Bio-Rad Mini-Protean II cell apparatus and electrophoresed according to the manufacturer’s instruction manual (Bio-Rad), and then the gels were immunooblotted as described above. For quantifying immunoblot results, the blots were scanned with an IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA).

**RESULTS**

**Spectral and Kinetic Properties of Recombinant NADH:NR**

To establish the utility of the *P. pastoris* expression system for producing plant NRs, the enzyme expressed in *P. pastoris* must be shown to be biochemically similar to NR isolated from plant tissues. To achieve this goal Arabidopsis NR from the NIA2 gene was expressed in liter-shake cultures of *P. pastoris,* partially purified, and analyzed. The time course of NR expression in *P. pastoris* shake cultures showed that the highest levels of enzyme activity were attained after 48 h of methanol induction (Fig. 2). The maximum NR level achieved after 48 h of induction was 200 units/L *P. pastoris* culture. NR was extracted from these cultures and purified in three steps (Table I). Proteins were precipitated with ammonium sulfate (45% AS pellet in Table I) and then purified by affinity chromatography on Blue-Sepharose, with NADH elution followed by binding to AMP-Sepharose and elution with NADH as described in “Materials and Methods.” The tight binding of NR to AMP-Sepharose is interesting, since we have observed that neither the recombinant Cyt b reductase nor the Cyt c reductase fragments of NR, which contain the NADH active site, binds tightly to this affinity gel (Shiraiishi and Campbell, 1997). These results suggest that holo-NR may have another binding site for AMP in addition to the NADH active site in the Cyt b reductase fragment of the enzyme. Recently, some evidence for the influence of AMP on regulation of NR was reported (Huber et al., 1996).

NR was purified to a specific activity of 36 units/mg protein, which represents more than a 550-fold enrichment compared with the crude extract (Table I). Analysis by SDS-PAGE of the purified NR revealed that it had a major protein band at approximately 110 kD and 10%, respectively. The samples were loaded onto a 5 to 20% gradient of Ready Gels for the Bio-Rad Mini-Protean II cell apparatus and electrophoresed according to the manufacturer’s instruction manual (Bio-Rad), and then the gels were immunooblotted as described above. For quantifying immunoblot results, the blots were scanned with an IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA).

**Spectral analysis** demonstrated the presence of absorbance peaks typical of plant NR (Fig. 3). The oxidized enzyme possesses a peak A443 that is characteristic of heme-Fe (Redinbaugh and Campbell, 1985; Campbell, 1991). Furthermore, reduction of the enzyme by the addition of solid NADH produced major peaks at 424 and 557 nm, which are characteristic of the internal Cyt b (heme-Fe) of NR (Redinbaugh and Campbell, 1985). Enzyme reduced with dithionite gave an identical spectrum (data not shown). These spectra compare favorably with the spectra previously reported for plant-derived NR (Solomonson et al., 1975; Redinbaugh and Campbell, 1985; Campbell, 1991). The A443/A413 ratio of 1.4 for the *P. pastoris*-derived NR is nearly identical to both squash and *Chlorella vulgaris* NR.
suggesting that the heme-Fe of these enzymes are structurally and functionally equivalent. There are some minor differences in the ratios of the UV absorbance to the Soret peaks at 413 and 424 nm. The *P. pastoris*-derived NR has ratios of 2.16 for $A_{280}/A_{413}$ and 1.52 for $A_{280}/A_{424}$, which are slightly higher than the ratios reported for squash and *C. vulgaris* NR (Solomonson et al., 1975; Redinbaugh and Campbell, 1985; Campbell, 1991). The ratios are approximately 15% higher and may be due to differences in the amino acid sequence (Campbell, 1991) or perhaps differences in sample preparation.

The kinetic properties of the *P. pastoris*-derived enzyme were also determined. Apparent $K_m$ values of 4 and 15 μM for NADH and nitrate, respectively, were determined. These kinetic constants are very similar to those previously reported for spinach NR (Barber and Notton, 1990). The *P. pastoris*-derived NR displays a pH optimum of 7.0, which is similar to previously characterized NR forms (Campbell and Smarrelli, 1978; Barber and Notton, 1990). Thus, by the criteria tested, NR produced in *P. pastoris* behaves like NR isolated from plants.

**Generation and Analysis of NR Mutants**

With the previous results giving us confidence that *P. pastoris*-expressed NR is similar to authentic plant NR, we next examined the function of several highly conserved amino acid residues through site-directed mutagenesis. One of the most important residues is the highly conserved Cys at position 191. This is the only invariant Cys residue in the MoCo-binding regions of both NR and sulfite oxidase, is essential for fungal NR activity, and is a proposed ligand to the molybdenum in NR (Barber and Neame, 1990; Solomonson and Barber, 1990; Garrett and Rajagopalan, 1994; Garde et al., 1995). Mutant forms of NR with alterations at position 191 would be invaluable for studying the role Cys-191 plays in binding molybdenum and catalyzing nitrate reduction in NR, as has been done for sulfite oxidase (Garrett and Rajagopalan, 1996). Mutations were constructed in the NR cDNA clone so that the Cys-191 was replaced with Ala or Ser.

Twenty *P. pastoris* transformants were generated for each mutation and then were tested for NR activity. NR activity was undetectable in extracts of *P. pastoris* expressing the C191S or C191A mutant forms of NR (Table II). In addition, bromphenol blue- and methyl viologen-dependent NR activities, which require a functional MoCo-binding region, were not detected in these extracts (data not shown). One explanation for the absence of NR activity in these extracts is that the altered NRs were not stable and did not accumulate in *P. pastoris*. To examine this possibility, immunoblot analyses were performed. Both C191A and C191S NR proteins were detectable in the *P. pastoris* extracts, although at a lower level than for wild-type NR (Fig. 4; Table II). Thus, the Cys-191 mutant NRs are present but inactive, indicating that Cys-191 is essential for nitrate-reducing activity.

Because native NR forms dimers (Solomonson and Barber, 1990), we wanted to determine whether the Cys-191 replacements affected dimerization. We investigated this by analyzing protein extracts on native gradient PAGE gels. Immunoblotting of the gels revealed that the mutant NR in the crude extracts forms both the apparent enzyme dimer and a dimer of dimers, as does wild-type NR in both crude extracts and purified form (Fig. 5). Therefore, Cys-191 is not required for NR dimerization, and its replacement with Ser or Ala does not perturb the protein structure of the monomer enough to impair dimer formation. It will be interesting to determine whether the MoCo is still bound in these altered forms of NR after they are purified and analyzed in more detail. In the case of sulfite oxidase, the MoCo is still bound to the modified enzyme in which the corresponding invariant Cys is replaced with a Ser (Garrett and Rajagopalan, 1996).

We next examined the importance of the N terminus on NR activity by altering the most N-terminal amino acids. Six residues (MAASVD/E) are highly conserved in plant NRs. Two mutants were generated that lacked this sequence: ntd3aa with a deletion of residues 2–4 (AAS) and ntd11aa with a substitution of residues 2 to 12 (AASVD-).
NRQYAR) with HHHHHHHHA (Fig. 1). The specific NR activities of these mutants were similar to wild-type NR (Table II). This result shows that these residues are not required for activity and suggests that it may be possible to add a His-tag sequence at the N terminus of NR to assist in purification. The N-terminal Met of Arabidopsis NR was replaced with MMHHHHHHHAS and transformed into P. pastoris. After induction, these strains produced functional NR at the same level as for wild-type NR (data not shown). NR containing the His-tag sequence could be partially purified to 4 units/mg specific activity in a single step using a bound-nickel column as described in “Materials and Methods.”

In only one known NR sequence from higher plants is the N-terminal sequence not conserved. This sequence is MSTCVEQ... from a maize NR genomic clone (GenBank accession no. U20450). This NR sequence also lacks the conserved hinge 1 sequence that is involved in the phosphorylation-dependent regulation of NR. Perhaps, the N-terminal sequence has been conserved along with the hinge 1 site for the proper regulation of NR. It has been suggested that the N-terminal 45 amino acids of spinach NR are needed for inhibition (Douglas et al., 1995). We assessed the role of the most N-terminal residues by assaying the ntd3aa, ntd11aa, and His-tag NR forms in our in vitro inhibition assay developed previously for Arabidopsis NR (Su et al., 1996). In this assay NR kinase and inhibitor protein are provided by an extract from an NR mutant of Arabidopsis called G'4-3 (Wilkinson and Crawford, 1993). After incubation with ATP recombinant NR provided by the P. pastoris extracts is inhibited. As can be seen in Table II and Figure 6, the two mutant forms of NR with 3 or 11 N-terminal amino acids missing were inhibited to the same extent as wild-type NR. Thus, the very N terminus of Arabidopsis NR is not essential for enzyme activity or the phosphorylation-dependent regulation.

![Figure 4](image)

**Table II. Comparison of wild-type (wt) and mutant NR expressed in P. pastoris**

<table>
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<tr>
<th>Enzyme Form</th>
<th>NR Crude Extract Specific Activity</th>
<th>NR Protein Loaded on PACE Gel</th>
<th>NR Protein from Western Blot</th>
<th>NR Specific Activity</th>
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<td>ng</td>
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<td>107</td>
</tr>
<tr>
<td>S365A</td>
<td>0.005</td>
<td>250</td>
<td>2.5</td>
<td>150</td>
</tr>
<tr>
<td>S395A</td>
<td>0.004</td>
<td>200</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>S438A</td>
<td>0.005</td>
<td>250</td>
<td>1.6</td>
<td>156</td>
</tr>
</tbody>
</table>

* Amount of NR protein for the wild-type was converted from a blot result to nanograms of NR protein using 100 units/mg as the specific activity for pure NR (see Redinbaugh and Campbell, 1985). 
* Calculated by dividing units of NR loaded onto gel by amount of NR protein determined by western blot.

![Figure 5](image)

Figure 5. Native gel analysis of wild type and C191 mutant NRs. Sample preparation and native gel electrophoresis were as described in “Materials and Methods.” Bio-Rad 5–20% polyacrylamide gradient gels were used. After electrophoresis, NR protein was visualized by immunoblot analysis as described in “Experimental Procedures.” In all lanes NR was obtained from P. pastoris extracts. Lanes 1 to 4, Wild-type NR; lane 5, C191A NR; and lane 6, C191S NR. The following amounts of protein and NR activity were added to each lane: lane 1, 100 µg of total protein (0.004 unit NR) of crude protein extract; lanes 2 and 3, 2 µg of protein (0.004 unit NR) and 1 µg of protein (0.002 unit NR) of partially purified wild-type NR (with a His-tag to aid purification, see “Materials and Methods”); lane 4, 50 µg of protein (0.002 unit NR) of crude protein extract; and lanes 5 and 6, 100 µg of protein of crude protein extract. The protein markers were thyroglobulin (669 kD), ferritin (440 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), and BSA (66 kD). Lanes 1 to 3 were produced on a gel different from that used for lanes 4 to 6. Arrows indicate corresponding bands on each gel.
Our last analysis focused on highly conserved Ser residues in the MoCo-binding region of NR: Ser-216, Ser-261, Ser-266, Ser-324, Ser-365, Ser-395, and Ser-438. These Ser residues could be essential for nitrate reduction or could be sites for NR phosphorylation, because it has been shown that NR is phosphorylated on multiple sites (Huber et al., 1992; Labrie and Crawford, 1994). These Ser residues were changed to Ala, and the mutant NR forms were expressed in P. pastoris, as described in “Materials and Methods.” Crude protein extracts from the P. pastoris transformants were assayed for NR activity and then separated by SDS-PAGE and visualized by protein blotting (Table II). All of the Ser-to-Ala-substituted NRs were functional and had no less than 45% of the wild-type level of NR activity after normalizing for the amount of NR protein in the extracts (Table II, last column). We conclude that none of these Ser residues are essential for NR activity: however, some (especially S266A, S365A, S395A, and S438A) may have an influence on NR function or stability. The Ser mutants were also tested in the in vitro inhibition assay. As shown in Figure 6, all mutant NRs showed Mg-dependent ATP inhibition similar to wild-type NR. Thus, none of these Ser residues are essential for the in vitro inhibition.

**DISCUSSION**

In this study we demonstrated that Arabidopsis NR produced in P. pastoris and purified to near homogeneity behaves like NR isolated from plants. Since NR has not been purified from Arabidopsis, we took advantage of the fact that NR has characteristic properties, regardless of the plant source from which it was isolated (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Hoff et al., 1994; Campbell, 1996). We found that the P. pastoris-derived NR has the same biochemical characteristics as natural plant NR. The visible spectra of oxidized and NADH-reduced enzyme and its kinetic characteristics are similar to those previously reported for NADH:Nr from squash, maize leaf, and C. vulgaris (Solomonson et al., 1975; Redinbaugh and Campbell, 1985; Hyde et al., 1989; Campbell, 1991). The maximal absorbances (at 413 nm in the oxidized and 424 and 557 nm in reduced enzyme) are virtually identical to those found for squash and corn holo-NR and the recombinant Cyt c reductase fragments of corn and spinach NR, all of which are highly similar to eukaryotic Cyt b6 (Redinbaugh and Campbell, 1985; Solomonson and Barber, 1990; Campbell, 1991, 1996). The P. pastoris-derived NR also has similar K_m values for both nitrate and NADH compared with plant-derived NR, being closest to those of spinach NR (Barber and Notton, 1990; Kleinhofs and Warner, 1990). The biochemical characteristics of the P. pastoris-derived NR described here, in addition to previous work (Su et al., 1996), make the P. pastoris expression system an attractive means to produce NR for more detailed biochemical characterization. Especially promising is the observation that under optimal methanol induction conditions in shake cultures (Fig. 2), P. pastoris produced more than 200 units/L active NR (estimated to be 2 mg/L of active NR based on a specific activity of 100 units/mg for pure NR [Redinbaugh and Campbell, 1985]).

Large quantities of pure enzyme will be needed for detailed biochemical and structural analysis of holo-NR, such as those that have been carried out with the recombinant fragments of NR (reviewed in Campbell, 1996). Whereas it is clear from our results that more NR can be obtained from P. pastoris than from plant sources, it is very likely that the expression of NR in P. pastoris can be improved even more. Even higher levels of NR production should be possible when P. pastoris is grown in a fermentor in which much higher cell densities can be achieved (Hagenson, 1991). We have also found that at least 5 times more spinach Cyt c reductase fragment than Arabidopsis holo-NR can be produced in P. pastoris (Shiraishi and Campbell, 1997). It is possible that MoCo production is limiting in P. pastoris, because we have found that supplementing the P. pastoris growth medium with molybdate

**Figure 6.** In vitro inactivation of wild-type (wt) and mutant NRs. P. pastoris extracts containing NR were mixed with desalted G3-3 extracts and then incubated with or without 5 mM ATP; an NR assay was then performed as described in “Materials and Methods.” The specific activity for each sample is shown in Table I. The values in the histogram are percentages of NR activity (NRA) relative to the 0 mM ATP control. Means and sds were calculated from duplicate experiments.
increases the production of active NR in P. pastoris (data not shown). These and other improvements are currently being investigated to increase the level of NR production in P. pastoris.

Availability of the P. pastoris expression system allowed us to investigate NR functionality using site-directed mutagenesis. A key residue of NR is Cys-191, which is the only invariant Cys among NRs and sulfite oxidase and has been proposed to provide a ligand to molybdenum in the MoCo-binding region (Barber and Neame, 1990; Solomonson and Barber, 1990; Garrett and Rajagopalan, 1994). Changing this residue to an Ala in Aspergillus NR completely abolishes NADPH:NR activity (Garde et al., 1995). Changing the corresponding Cys in rat and human sulfite oxidase to Ser reduces oxidase activity by more than 2000-fold and changes the spectra of the MoCo-binding region (Garrett and Rajagopalan, 1996). Mutating the corresponding Cys (Cys-191) in Arabidopsis NR abolished activity, showing that this residue also plays an essential role in plant NRs (Table II). Because this mutation did not prevent the accumulation of NR in P. pastoris (Fig. 4), we then asked whether it affected the quaternary structure of NR. It has been shown that NR forms dimers and dimers of dimers (Redinbaugh and Campbell, 1985; Hyde et al., 1989; Solomonson and Barber, 1990; Truong et al., 1991). Changing this residue to an Ala in Arabidopsis NR altered at position Cys-191 apparently still forms native mutant forms of NR in which residues 2 to 4 (ntd3aa) are conserved, and yet they have no known function. We examined native mutant forms of NR in which residues 2 to 4 (ntd3aa) were deleted and residues 2 to 12 were substituted with a His-tag sequence (ntd11aa). Neither mutant was impaired in NR activity or ATP-dependent inhibition. These results encouraged us to alter the N terminal of NR by adding a His-tag sequence directly to the N terminus of NR, providing a rapid purification of NR using metal-affinity chromatography. We also replaced a11 of the conserved Ser residues in the MoCo-binding region of Arabidopsis NR and found that none were required for NR activity or ATP-dependent inhibition. Taken together with our previous work, these results refine our understanding of NR structure and regulation and open up new avenues for the study of this important enzyme in plant nitrogen metabolism.

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