Expression in *Escherichia coli* of Cytochrome c Reductase Activity from a Maize NADH:Nitrate Reductase Complementary DNA

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ABSTRACT

A cDNA clone was isolated from a maize (*Zea mays* L. cv W64AXW183E) scutellum λgt11 library using maize leaf NADH:nitrate reductase Zmr1 cDNA clone as a hybridization probe; it was designated Zmr1S. Zmr1S was shown to be an NADH:nitrate reductase clone by nucleotide sequencing and comparison of its deduced amino acid sequence to Zmr1. Zmr1S, which is 1.8 kilobases in length and contains the code for both the cytochrome *b* and flavin adenine dinucleotide domains of nitrate reductase, was cloned into the *EcoRI* site of the *Escherichia coli* expression vector pET5b and expressed. The cell lysates contained NADH:cytochrome c reductase activity, which is a characteristic partial activity of NADH:nitrate reductase dependent on the cytochrome *b* and flavin adenine dinucleotide domains. Recombinant cytochrome c reductase was purified by immunoadfinity chromatography on monoclonal antibody Zm2(69) Sepharose. The purified cytochrome c reductase, which had a major size of 43 kilodaltons, was inhibited by polyclonal antibodies for maize leaf NADH:nitrate reductase and bound these antibodies when blotted to nitrocellulose. Ultraviolet and visible spectra of oxidized and NADH-reduced recombinant cytochrome c reductase were nearly identical with those of maize leaf NADH:nitrate reductase. These two enzyme forms also had very similar kinetic properties with respect to NADPH-dependent cytochrome c and ferricyanide reduction.

Higher plant NADH:NR\(^2\) (EC 1.6.6.1) catalyzes the first step in nitrate assimilation and is a key site for regulation of this pathway (22). The enzyme is a homodimer composed of two approximately 100-kDa polypeptide chains with each containing one equivalent of FAD, heme-Fe, and Mo-pterin (2, 3, 17). Each of these electron-carrying cofactors appears to be bound into a specific region of the enzyme's polypeptide, which folds into distinct domains. The N-terminal region of NR can be recognized as the Mo-pterin domain by comparing the deduced amino acid sequences of NR to related mammalian protein sequences because of its similarity to the Mo-pterin domain of sulfite oxidase, and the C-terminal region of NR has sequence homology to NADH:Cyt *b* reductase, which is a FAD-containing enzyme (3, 22). In between the Mo-pterin and FAD domains, NR has similarity to mammalian Cyt *b*\(_2\). This central Cyt *b* domain of NR is bridged to the other two domains on either side of it by highly variable sequence regions that appear to be hinges (3). These hinge regions are probably exposed in native NR and susceptible to attack by proteolytic enzymes (15, 22). Hence, native NR can readily be cleaved by proteinases into stable fragments that retain partial catalytic activities of holo-NR, such as methyl viologen NR, NADH:Cyt *c* reductase, or NADH:ferricyanide reductase (15, 22). The stable fragments appear to be the domains of the native NR or combinations of two domains; for instance, the fragment with methyl viologen NR activity is composed of the Mo-pterin and Cyt *b* domains, the Cyt *c* reductase fragment contains the Cyt *b* and FAD domains, and the ferricyanide reductase fragment containing the FAD domain (3, 15, 22).

These catalytically active and stable fragments of NR have been useful in sorting out some of the complexities of the biochemistry of NR (2, 3, 22). However, it is difficult to produce these fragments in sufficient amounts for many studies, especially those involving physical and chemical measurements.

An alternative approach is to produce the NR domains and their catalytically active combinations as recombinant proteins in *Escherichia coli* using cDNA clones for NR. The FAD domain of maize leaf NADH:NR was overexpressed in *E. coli* using part of the Zmr1 cDNA (9) and purified to homogeneity by NADH elution from blue Sepharose (11). The recombinant FAD domain of NR has an *M*\(_r\) of 30,000, NADH:ferricyanide reductase activity, and a UV-visible spectrum essentially identical with mammalian NADH:Cyt *b* reductase, which demonstrated that FAD is bound to the recombinant protein in a functional manner (11). The recombinant FAD domain has now been crystallized, and analysis of its three-dimensional structure is underway (G. Lu, W.H. Campbell, Y. Lindqvist, and G. Schneider, unpublished results). The Cyt *b* domain of *Chlorella* NADH:NR has also been expressed in *E. coli*, and although this domain has no catalytic activity by itself, the recombinant protein has a visible spectrum very similar to native NR (5).

Reported here is the expression in *E. coli* of a fragment of

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2. Abbreviations: NR, nitrate reductase; anti-FD, rabbit polyclonal antibody for SDS-denatured recombinant FAD domain of NR; anti-NR, rabbit polyclonal antibody for nitrate reductase; FAD, flavin adenine dinucleotide; IgG, immunoglobulin G; IPTG, isopropyl β-D-thiogalactopyranoside.
NADH:NR with NADH:Cyt c reductase activity. The recombinant protein was purified using monoclonal antibody-based immunoaffinity chromatography, which has been used for purification of holo-NR from maize leaf extracts (12). Recombinant Cyt c reductase has immunohemochromatic, kinetic, and spectral properties virtually identical with NADH:NR purified from maize leaves by the same method.

MATERIALS AND METHODS

Isolation of Maize Scutellum cDNA Clones for NR

Total RNA was isolated from nitrate-induced scutella of maize (Zea mays L. cv W64A×W183E) seedlings and shown to contain a transcript that hybridized to the Zmnr1 cDNA clone, as previously described (10). cDNA was synthesized from polyadenylated RNA isolated on mAP paper (Amersham, Chicago, IL) and cloned into EcoRI-cut λgt11 arms (Stratagene, San Diego, CA) after EcoRI-NotI linker-adaptor (GAATTCGCGGCCGC) ligation (Libarian cDNA cloning kit; Invitrogen, San Diego, CA). The maize scutellum cDNA library was screened by hybridization with radioactive Zmnr1 insert DNA, and four positive clones were isolated and plaque purified (9, 18). Insert DNA was excised with EcoRI and subcloned into the EcoRI site of Bluescript II vector (Stratagene). Nucleotide sequencing was carried out by the dideoxy-nucleotide method on the double-stranded DNA of these subclones using T7, T3, SK, and KS primers (Stratagene), as well as six custom primers derived from the nucleotide sequence of Zmnr1 (9). One of these clones, designated Zmnr1S, is very similar to Zmnr1 in nucleotide sequence and contains 1804 base pairs, which makes it 300 base pairs shorter than Zmnr1 (9). The other three clones were either smaller or contained some nucleotide sequence regions not related to Zmnr1.

Construction of the Expression Vector

Zmnr1S insert was excised with EcoRI and cloned into the EcoRI site of the E. coli expression vector pET5b (Novagen, Madison, WI). After the clone was transformed into E. coli strain DH5α- competent cells, three clones were selected containing the Zmnr1S insert, and the orientation was determined by nucleotide sequencing using the T7 primer, which binds to the T7 promoter in the pET vector series (24). Two of the clones were in frame with the AUG translation start site of the pET5b vector, designated pZCR1 and pZCR2, and the other was in the opposite orientation and designated pZCR3.

Expression of Zmnr1S in E. coli

The constructs pZCR1, pZCR2, and pZCR3 were transformed into E. coli strain BL21(DE3)pLysS-competent cells, and colonies harboring both the expression plasmid and the pLysS plasmid were selected by restriction analysis (18, 24). The cells were grown in LB medium or LBM9 medium modified by the addition of 0.14 mm Fe-citrate, with both containing 20 μM ampicillin (24). Cells were grown at 37°C, except where noted otherwise. To express the Cyt c reductase fragment encoded by the insert DNA, cells were induced with 0.4 μM IPTG to express the T7 polymerase, which resides in the chromosome of the DE3 cell strain and is under the control of the inducible lac UV5 promoter (24). Cells were grown to 0.6 A600nm before induction with IPTG. After 2.5 to 3 h of induction with IPTG, the cells were harvested by centrifugation.

Purification of Recombinant Cyt c Reductase

E. coli cells harvested from two 1-L cultures were suspended in 1 L of 0.1 m KPO4 (pH 7.5) and 1 mM EDTA and frozen at −70°C for at least 12 h before thawing. No additives were required to lyse the cells because they were expressing T7 lysozyme from the pLysS plasmid (23). After thawing, cell debris was removed by centrifugation for 30 min at 20,000 g, and the supernatant fluid was mixed with 7 to 10 mL of Zm2(69) Sepharose (monoclonal antibody Zm2(69) coupled to cyanogen bromide-activated Sepharose 4B [Pharmacia] at 1 mg of IgG/mL gel) at 4°C for 45 min (12). The gel was recovered by vacuum filtration and washed with a total of 2 L of suspension buffer using three washes with the gel collected by filtration in between each wash. The washed gel was mixed with a minimum volume of suspension buffer and packed into a column (0.9 × 15 cm). The column was eluted with 10 mM glycine (pH 11), and 1.1-mL fractions were collected into tubes containing 15 μL of 2 M NaPO4, pH 7 (12). Crude extract, the first filtrate obtained immediately after the binding step, and purified fractions eluted from the immunoaffinity column were assayed for Cyt c reductase activity (21), protein (16), and A415 nm (purified fractions only).

Purification of Other Enzyme Forms and Miscellaneous Methods

Previously described methods were used to purify maize leaf NADH:NR (12) and the recombinant FAD domain (11). Spectra of purified Cyt c reductase and maize leaf NADH:NR were analyzed with a Shimadzu UV-1201 UV/visible spectrophotometer at 25°C. These proteins were reduced with solid NADH to generate their reduced spectra. Kinetic analyses were done at 25°C with a Gilford 2400–2 UV/visible spectrophotometer measuring the change in absorbance with time for Cyt c reductase at 550 nm and for ferricyanide reduction at 340 nm (NADH), which were converted to micromoles per minute using the following millimolar extinction coefficients: NADH, 6.2 (340 nm); Cyt c, 20 (550 nm). SDS-PAGE and protein blotting to nitrocellulose were done as previously described (4, 17). Polyclonal antibodies for SDS-denatured recombinant FAD domain of maize leaf NADH:NR were elicited in a rabbit using two subcutaneous injection sets, 3 weeks apart, with 1 mg of protein for each injection set, as previously described for preparation of anti-NR (4). The rabbit was exsanguinated 10 d after the second injection set. Anti-NR, anti-FD, and monoclonal antibody Zm2(69) were the purified IgG fractions obtained from a protein-A Sepharose column (4). The specificity of the anti-NR was previously described (4). Protein blots were developed with anti-rabbit IgG conjugated to alkaline phosphatase (Cappel, Organon Teknika Corp, West Chester, PA) using a standard alkaline phosphatase blot assay (9).
RESULTS AND DISCUSSION

Construction of the Expression Vector

When Zmnr1S\(^3\) insert is excised with EcoRI and cloned into the EcoRI site of the pET5b expression vector, Zmnr1S would be inframe with the translation initiation codon of the vector (24). This construct, designated pZCR1, was made, and the predicted alignment was confirmed by nucleotide sequencing. When expressed in *E. coli*, pZCR1 is predicted to yield a polypeptide with 520 amino acid residues and an *M*\(_s\) of 58,067. The N-terminal 18 residues would be derived from the vector and the EcoRI-NotI linker-adaptor of the insert, and the remaining 502 residues would be from Zmnr1S, except for the first nucleotide of residue 19 being contributed by the last nucleotide of the linker-adaptor (Fig. 1A). The amino acid sequence deduced from Zmnr1S differs from the sequence of Zmnr1 (9) in 18 residues (data not shown). In most cases, these differences result from single-base changes in the nucleotide sequence of Zmnr1S relative to the Zmnr1 sequence (9), except near the 5' end, where three nucleotides are inserted, and in the FAD domain, where a nine-nucleotide insertion is found.

Interestingly, most of these substitutions in the deduced amino acid sequence of Zmnr1S relative to Zmnr1 (9) result in residues found in either the rice or barley NR-deduced amino acid sequences (6, 7, 19). Because these maize NR cDNA clones have virtually the same 3'-untranslated nucleotide sequence, Zmnr1 and Zmnr1S appear to be alleles rather than separate genes for NADH:NR; however, this is not completely established. Based on an restriction fragment length polymorphism map of segregation data pooled from four *F*\(_2\) populations of maize inbred lines, Zmnr1 maps to two loci: one on chromosome 2 near the centromere and a second undetermined location (D. Grant, personal communication). Consequently, further analysis is required to establish the relationship between Zmnr1 and Zmnr1S. Because these two cDNA clones were isolated from different cultivars of maize, one of which is an inbred line (9) and the other a hybrid of two inbred lines apparently unrelated to the first, they may represent the degree of natural variation occurring in the maize NADH:NR gene.

The 58-kD protein predicted to be expressed from pZCR1 would contain the complete Cyt b and FAD domains of NADH:NR, as well as a portion of the Mo-pterin domain (3, 9, 17, 22). Based on the amino acid sequence alignments of all available NR sequences and related proteins (3, 22), the Cyt b domain of the predicted recombinant protein encoded by pZCR1 would begin at residue 151 and end at 226 (Fig. 1B, 76 residues total), and the FAD domain would begin at residue 259 and end at the natural C terminus shared by all NADH:NR forms (Fig. 1C, 262 residues total). The hinge region between these two domains appears to be 33 residues long, but the assignments of the C terminus of the Cyt b domain and the N terminus of the FAD domain are only approximations. Because both the Cyt b and FAD domains of NR have been expressed separately in *E. coli* (5, 11), it can be expected that the Cyt b and FAD domains of the recombinant Zmnr1S protein would bind compatible cofactors and fold in stable conformations, which might protect the hinge between them against degradation.

However, the N-terminal 150 residues of the recombinant protein would not be expected to bind the Mo-pterin cofactor synthesized in *E. coli*, which differs from that found in maize NR (8, 13). The N-terminal region might fold into a random coil, which may be susceptible to proteolysis, especially in the hinge region between the incomplete Mo-pterin domain and the stably formed Cyt b domain (Fig. 1D). This region of NR has been shown to be cleaved by trypsin when spinach NADH:NR is subjected to mild proteolysis (15), and it is cleaved at the same site shown in Figure 1D as a potential proteolytic cleavage site in the recombinant Zmnr1S protein (8). The larger polypeptide fragment resulting from such a cleavage of the 58-kD Zmnr1S recombinant protein would be predicted to have an *M*\(_s\) of 42,797 or molecular mass of 43 kD.

**Cyt c Reductase Activity Expression in *E. coli***

Lysates of *E. coli* cells containing pZCR1, grown at 37°C in LB medium and induced with IPTG, were prepared using a single freeze/thaw cycle, and after centrifugation, the supernatant fluid was found to have Cyt c reductase activity. A single freeze/thaw cycle is sufficient to break the cell walls of *E. coli* when the cells express the pLysS plasmid, which encodes T7 lysozyme (23). Thus, it appeared that pZCR1 was being expressed in *E. coli* to yield an active Cyt c reductase fragment of NR; however, the level of expression was not as high as had been achieved with the recombinant FAD domain.

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\(^3\) The nucleotide sequence of Zmnr1S has been deposited in GenBank and assigned accession No. M77792.
(11). Studier et al. (24) suggested that higher levels of expression may be obtained in an enriched medium containing glucose. Others using the pET vector system for expressing heterologous proteins in E. coli found more protein of interest in the soluble fraction when a lower growth temperature was utilized (20). The highest specific activity was found in the enriched LB9 medium when cells were grown at 37°C, as compared to growth in LB medium. Growth at 32°C in either the enriched LB9 or LB media did not enhance the production of the recombinant Cyt c reductase activity. A second transformant of E. coli containing an inframe construct, pZCR2, was also analyzed for Cyt c reductase activity and was found to have a similar level to the pZCR1 transformant. A transformant harboring a construct with the insert DNA in the opposite orientation, pZCR3, was used as a negative control in these analyses. The factors limiting the expression of the recombinant Cyt c reductase activity remain to be established.

**Purification of Recombinant Cyt c Reductase**

When an E. coli lysate with expressed Cyt c reductase activity was mixed with blue Sepharose and the gel washed to remove nonbinding proteins, the activity was completely bound. However, NADH did not elute the Cyt c reductase activity, but it was eluted with 1 M NaCl (data not shown). This procedure yielded a low specific activity Cyt c reductase, which contained a large number of protein bands on SDS-PAGE. An effective purification was achieved using anti-NR monoclonal antibody Zm2(69) Sepharose with elution at pH 11, which is a method for purification of maize leaf NADH:NR (12). Cyt c reductase activity was eluted from the Zm2(69) Sepharose in a sharp peak, which absorbed light at 413 nm, a characteristic of the heme-Fe cofactor of NR (Table I). The specific activity of the Cyt c reductase activity was increased 800-fold in the peak fraction (fraction 7) of the Zm2(69) Sepharose pH 11 elution, in which 60% of the activity bound to the column was recovered (Table I). When NADPH was substituted for NADH in the Cyt c reductase assay, the purified recombinant enzyme was slightly more active than maize leaf NADH:NR with this substrate but clearly had a strong preference for NADH (Table II).

SDS-PAGE analysis of fractions 7 and 8 from the Zm2(69) Sepharose column revealed that both of these high specific activity Cyt c reductase fractions contained a series of polypeptides ranging in size from 58 to 43 kD with the largest amount appearing to be 43 kD, especially in fraction 8 (Fig. 2A, lanes 4 and 5). For comparison, the recombinant FAD domain of maize NR and maize leaf NADH:NR, 31 and approximately 100 kD (2, 11), respectively, were also included in this gel (Fig. 2A, lanes 3 and 6). When a similar gel was electrophoresed with these same proteins and transferred to nitrocellulose, polyclonal anti-NR bound to all of the polypeptides in fractions 7 and 8 (Fig. 2B, lanes 2 and 6, respectively), as well as the FAD domain (Fig. 2B, lane 1) and holo-NR (Fig. 2B, lanes 3 and 5). These results indicate that the full size 58-kD polypeptide predicted to be made from the target sequence in pZCR1 is expressed, but this polypeptide is unstable in the cells and/or lysate. It appears to be degraded in steps with at least two intermediate polypeptides and a stable 43-kD polypeptide accumulating as the final product. Inclusion of PMSF in the lysis buffer did not prevent the degradation of the 58-kD polypeptide (data not shown). Although this size heterogeneity of the recombinant Cyt c reductase complicates its biochemical analysis, the impact of

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<th>Table I. Purification of Recombinant Cyt c Reductase by Monoclonal Antibody-Based Immunopurification Chromatography Using Anti-NR Zm2(69) Sepharose</th>
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* NADH: Cyt c reductase activity units = μmol Cyt c reduced/min.  
  * ND, Not determined.

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<th>Table II. Comparison of Cyt c Reductase Activity of Recombinant Cyt c Reductase and Maize Leaf NADH:NR in the Presence of NADPH or Antibodies</th>
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* NADH was omitted from this assay and replaced by 0.1 mM NADPH.
FAD domain of maize NR (Table II). All three antibodies inhibited the Cyt c reductase activity of the recombinant enzyme and maize leaf NADH:NR. Anti-NR was by far the most inhibitory with a greater effectiveness for the holoenzyme than the recombinant fragment, whereas anti-FD and Zm2(69) were slightly more effective inhibitors of the fragment (Table II). Anti-FD is a weak inhibitor and adding increasing amounts of it did not enhance its effectiveness, whereas the degree of inhibition produced by anti-NR and Zm2(69) was highly concentration dependent (data not shown). SDS-PAGE gels of the proteins in lysates of E. coli expressing the FAD domain and Cyt c reductase and the purified FAD domain and Cyt c reductase were transferred to nitrocellulose and developed with anti-FD. Although there were many proteins present in the lysates (Fig. 3A, lanes 1 and 2), the anti-FD bound only to a 30-kD protein in a lysate from cells expressing the FAD domain, which establishes the specificity of this antibody (Fig. 3B, lane 1). However, although the anti-FD bound to purified Cyt c reductase polypeptides (Fig. 3B, lane 4), no antibody binding was detected in the lysate of cells expressing them, which may be due to their low concentration in the lysate (Fig. 3B, lane 2). Anti-FD also binds to purified holo-NR from maize leaves in protein blots (Fig. 3B, lane 5). These tests leave little doubt

this complexity was minimized by using spectra to evaluate the heme-Fe content, which was taken to represent the fraction of the protein mixture containing a functional enzyme form.

Immunological Analysis of Recombinant Cyt c Reductase

As described above, recombinant Cyt c reductase binds to monoclonal antibody Zm2(69), and polyclonal anti-NR binds to its denatured polypeptides. Because these two antibody preparations have been shown to be specific for NR (4, 12), the Cyt c reductase activity expressed was encoded by pZCR1 and has epitopes in common with maize NADH:NR. The immunological character of recombinant Cyt c reductase was further delineated with these two antibodies and a polyclonal antibody prepared against the denatured recombinant

Figure 2. SDS-PAGE analysis of recombinant Cyt c reductase and protein blotting with anti-NR. A, A 10% acrylamide SDS-PAGE gel was used for separation of the polypeptides and stained with Coomassie blue. Lane 1, High molecular mass standard proteins of 200, 116.25, 97.4, 66.2, and 45 kD (Bio-Rad); lane 2, low molecular mass standard proteins of 97.4, 66.2, 45, and 31 kD; lane 3, purified recombinant FAD domain of NR (11); lane 4, fraction 7 of the immunoadfinity-purified recombinant Cyt c reductase (Table I); lane 5, fraction 8 (Table I); lane 6, purified maize leaf NADH:NR (12). B, A 12% acrylamide SDS-PAGE gel was used to separate the polypeptides, which were electrophoretically transferred to supported nitrocellulose membrane (OPTIBIND, Schleicher and Schuell) and developed with anti-NR (4) followed by binding of antibody to rabbit IgG conjugated to alkaline phosphatase and staining for enzyme activity bound to the polypeptides (9). Lane 1, Purified recombinant FAD domain of NR (11); lane 2, fraction 7 (Table I); lanes 3 and 5, purified maize leaf NADH:NR (12); lane 4, RAINBOW protein molecular mass markers (Amersham) used to calibrate blot but not visible in photograph; lane 6, fraction 8 (Table I). The protein blot contained artifacts of unknown origin at 60 and 70 kD in all lanes, including the RAINBOW standards. The purified maize leaf NADH:NR contained a small amount of 85-kD protein, which bound anti-NR and is presumed to be a degradation product of purified 100-kD NR polypeptide.

Figure 3. SDS-PAGE analysis of E. coli lysates and recombinant Cyt c reductase and protein blotting with anti-FD. A, A 12% acrylamide SDS-PAGE gel was used for separation of the polypeptides and silver stained (Bio-Rad). Lane 1, Lysate of E. coli cells expressing recombinant FAD domain of NR (11); lane 2, lysate of E. coli cells expressing recombinant Cyt c reductase; lane 3, purified recombinant FAD domain (11); lane 4, purified recombinant Cyt c reductase from a different Zm2(69) Sepharose column than described in Table I; lane 5, purified maize leaf NADH:NR (12); lane 6, combined high and low molecular mass standard proteins of 116.25, 97.4, 66.2, 45, 31, and 21.5 kD (Bio-Rad). B, Identical gel to A transferred to nitrocellulose and developed with anti-FD as in Figure 2B; lane assignments are as in A, except standards were omitted. The blot for lane 5 containing maize leaf NADH:NR was from a separate gel and appeared to contain degradation products of holo-NR at 43 and 30 kD, which bound anti-FD. RAINBOW protein markers were used to calibrate the blots.
that recombinant Cyt c reductase is immunochemically related to NR and its recombinant FAD domain. This is to be expected because these proteins are predicted to have >95% identity in their common amino acid sequence portions.

**Spectral and Kinetic Analyses of Recombinant Cyt c Reductase**

Spectra were taken for the oxidized and NADH-reduced Cyt c reductase and compared with those of purified maize leaf NADH:NR (Fig. 4). In the visible range, both proteins have major peaks at 413 nm in the oxidized form and 557 and 424 nm in the reduced form. However, they differ in the UV with Cyt c reductase peaking at 265 nm, and NR peaking at 275 nm and having a broader absorbance, which may be due to the presence of the Mo-pterin cofactor. These spectra are very similar to those of squash NADH:NR and Chlorella NADH:NR and its recombinant Cyt b domain (5, 17). The major differences are in the ratios of the UV absorbance to the Soret peaks (413 and 424 nm), with the ratios for maize leaf NADH:NR (R₁ = A₂80nm/A₄13nm = 2.10; R₂ = A₂80 nm/ A₄24 nm = 1.67) being greater than those for squash (R₁ = 1.79; R₂ = 1.26) or Chlorella NADH:NR (R₁ = 1.84; R₂ = 1.45); the recombinant Cyt c reductase has lower ratios (R₁ = 1.32; R₂ = 1.02). The small differences in the spectral properties of the NADH:NR forms are probably due to differences in amino acid sequence and, in the case of the maize leaf NR, due to the pH 11 treatment during purification, which results in decreased NADH:NR activity (12). The much lower ratios for recombinant Cyt c reductase are likely due to the absence of the Mo-pterin cofactor. The ratios of the peaks in the visible portion of the spectra, which can be attributed to the heme-Fe of their Cyt b domain, are similar for all four proteins, indicating that the environment of the heme-Fe is similar in each of these proteins.

The kinetic properties of the recombinant Cyt c reductase were compared to those of maize leaf NADH:NR and the recombinant FAD domain (Table III). Because the FAD domain contains only FAD as an internal cofactor and cannot reduce Cyt c (11), the kinetics of ferricyanide reduction were compared for these three enzyme forms and found to be virtually identical. The recombinant Cyt c reductase has a slightly higher Km (Cyt c) and lower Vmax for Cyt c reduction than maize leaf NADH:NR (Table III). These kinetic constants for partial reactions of NR are very similar to those for Chlorella and spinach NADH:NR (1, 14, 22).

**Summary**

A new cDNA clone for maize NADH:NR was used to express a protein in E. coli with Cyt c reductase activity. The protein was isolated in a partially degraded form but displayed spectral and kinetic properties consistent with it having a stable combination of the Cyt b and FAD domains of NADH:NR. The recombinant Cyt c reductase shares epistopes with native and denatured maize leaf NADH:NR, as well as the recombinant FAD domain of this enzyme. Although the recombinant Cyt c reductase was not expressed at a high level, the system will be useful for determining essential amino acid residues of the Cyt b and FAD domains of NR via site-directed mutagenesis, especially when studied in combination with the recombinant FAD domain which is expressed at a high level (11).

![Figure 4. Spectra of recombinant Cyt c reductase (CCR) and maize leaf NADH:NR. The proteins were purified by pH 11 elution from Zm2(69) Sepharose. Oxidized (— — —) and reduced (- - -) spectra were taken at 25°C as described in "Materials and Methods." Proteins were reduced with solid NADH.](image-url)
EXPRESSION OF CYT c REDUCTASE OF MAIZE NITRATE REDUCTASE

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LITERATURE CITED


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