Expression and Characterization of the Heme-binding Domain of Chlorella Nitrate Reductase*

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A recombinant protein corresponding to the putative heme-binding domain of assimilatory NADH:nitrate reductase from Chlorella vulgaris has been expressed and purified from transformed Escherichia coli BL21 cells. The recombinant protein, exhibited a subunit molecular mass of approximately 10 kDa with a N-terminal sequence beginning with the residues PAGA in agreement with that predicted by cDNA analysis. The UV-visible spectrum of the protein confirmed the incorporation of heme with maxima at 413 nm and 423, 528, and 557 nm for the oxidized and reduced forms, respectively. Circular dichroism spectra indicated the environment of the heme chromophore was very similar to that of the native enzyme. Potentiometric titrations of the recombinant heme domain yielded a midpoint potential of +16 mV (n = 1, pH 7), substantially higher than the values of -160 mV obtained for the native enzyme and -28 mV obtained for a previously expressed recombinant heme domain that contained part of the Mo-pterin domain. These results indicate that portions of the amino acid sequence that are involved in the formation of the Mo-pterin domain of Chlorella nitrate reductase influence the redox potential of the heme prosthetic group.

Nitrate assimilation, the eight-electron reduction of nitrate to ammonium in eukaryotes is catalyzed by two metalloenzymes, nitrate reductase (NR)1 and nitrite reductase (NiR) (1) according to the scheme as follows,

\[
\begin{align*}
\text{NO}_3^- & \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+ \\
\text{NAD(P)}H & \rightarrow \text{NAD}^+ \rightarrow \text{Fd}^- \rightarrow \text{Fd}
\end{align*}
\]

Scheme I

where NAD(P)H and reduced ferredoxin function as the respective physiological electron donors for these two enzymes (2-3). The initial step in the pathway, the reduction of nitrate to nitrite, is considered to be the regulated step in nitrate assimilation and a rate-limiting factor in the growth and development of nitrate-assimilating organisms (1).

In the unicellular green alga, Chlorella vulgaris, NR exists as a homotetramer of subunits (molecular mass 100 kDa) each of which contains FAD, a bs-type cytochrome and Mo-pterin in a 1:1:1 stoichiometry (1). The roles of these prosthetic groups have been extensively defined using steady-state kinetics (4), potentiometric studies (5), limited proteolysis (6), and radiation inactivation analysis (7). The FAD and Mo-pterin domains function as the binding sites for NADH and NO3--, respectively (8), while the bs cytochrome has been proposed to mediate the intramolecular transfer of reducing equivalents from the FAD to the Mo-pterin (9). This electron transfer pathway has been derived from a combination of thermodynamic measurements and electrochemical analysis of the various partial activities exhibited by the enzyme (1).

Radiation inactivation (7) and limited proteolysis (6) have suggested that these partial activities are associated with functionally independent domains and did not appear to be influenced by or require prosthetic group centers not directly required for the particular NR partial activity.

The heme midpoint potential (E^' = -160 mV, n = 1) of the native enzyme has been shown to be intermediate between the potentials of FAD (E^' = -272 mV, n = 2) and Mo-pterin (E^' = -10 mV, n = 2) (5). Similar values for the heme potential have been obtained for other NRs isolated from fungi (9) and higher plants (10). The low negative potential for the NR heme contrasts significantly with the more positive values obtained for other bs-type cytochromes such as mossominal cytochrome bs (11), yeast flavocytochrome bs (11), and hepatic sulfite oxidase (E^' = +5 mV) (11) despite the fact that these proteins exhibit significant amino acid sequence similarity within their respective heme-containing domains. In addition, invariant residues postulated to have structural or functional significance are present in all of these physiologically unrelated proteins (13).

In order to define factors influencing the unusually low heme midpoint potential of NR, we have expressed and characterized a recombinant Chlorella NR heme domain comprising the minimal portion of the enzyme's primary sequence necessary for the production of this functional domain.

**MATERIALS AND METHODS**

*Expression and Purification of the Heme-binding Domain—Two expression clones were constructed which coded for the heme-binding domain of NR. The first clone, pET 3bNR was constructed as previously described (14) by inserting the pCVNR1 clone (Fig. 1) into the IPTG-inducible pET 5b expression vector. The recombinant protein expressed by this clone was purified by ammonium sulfate fractionation (40-75%), followed by FPLC using a Mono-Q column (Pharmacia LKB Biotechnology Inc.). The protein was eluted using a salt gradient (0-500 mM NaCl), desalted by dialysis, and concentrated. The purified protein was estimated to be approximately 50% pure by SDS-PAGE and exhibited an A280/A415 of < 1.5.*

The second clone constructed coded specifically for the minimal bs-cytochrome-binding domain as determined by alignment of the
amino acid minimal heme-binding domain. U.P. refers to the upCVNR1, was denatured at 94°C, followed by annealing at 50°C and Amplification of the domain was achieved by polymerase chain reaction at 72°C. This cycle was repeated 30 times. The pH range of 4-10 using a Bio-Rad IEF Rotofor (Melville, NY). The stream primer, sequence for synthesis of the domain. The core cDNA (300 bp) encodes the 92- and main and was inserted into the EcoRI site of pET5b expression vector. Correctly expressing the heme domain were identified by growth at and following 2 h growth at 37°C, expression was assessed by determining the absorbance of the cells over the 400-500-nm wavelength range to confirm the presence of induced heme production. Protein, expressed cells (2 h after IPTG addition) were harvested, resuspended in buffer, and disrupted in a French pressure cell (15,000 psi.). The clarified homogenate was subjected to preparative isoelectric focussing over a pH range of 4-10 using a Bio-Rad IEF Rotofor (Melville, NY). The domain was further purified by FPLC using ion-exchange (Mono-Q) and gel filtration. Purity of this protein, assessed by SDS-PAGE, was estimated at approximately 80% with an A280/A450 of < 0.3. For amino acid sequencing studies, the pETANR1 heme domain was further purified by reversed-phase HPLC as previously described (16).

FIG. 1. Schematic of cDNAs encoding NR heme-binding domains. pCVNR1 (1 kilobase) encodes the 35-kDa recombinant domain and was inserted into the EcoRI site of pET5b expression vector for synthesis of the domain. The core cDNA (300 bp) encodes the 92-amino acid minimal heme-binding domain. U.P. refers to the upstream primer, sequence 5' GGCGATCCGGCGGACGTGCCAACAGAC G3'. D.P. refers to the downstream primer, sequence 5'GGCGATCCC GGTGCCCGCGTGCTCGGC3'. U.P. and D.P. primers were used to generate the 300-bp cDNA by polymerase chain reaction, which was then subcloned into the expression vector pET3a for synthesis of the 10-kDa recombinant domain.

known portions of the Chlorella NR primary sequence with that of calf cytochrome b6 (13). Specific primers, shown in Fig. 1, were designed for amplification of a 300-bp fragment coding for the "core" heme-binding domain with the introduction of NdeI and BamHI restriction sites at the 5'- and 3'-ends of the sequence, respectively. Amplification of the domain was achieved by polymerase chain reaction (15) using Taq polymerase. Linearized template DNA, pCVNR1, was denatured at 94°C, followed by annealing at 50°C and chain extension at 72°C. This cycle was repeated 30 times. The inclusion of 5% dimethyl sulfoxide in the reaction buffer considerably improved the specific amplification of a 300-bp product. Following restriction digestion using NdeI and BamHI, the amplified DNA was inserted into the NdeI: BamHI site of the pET3a expression vector and transformed into Escherichia coli BL21 cells. Clones that were correctly expressing the heme domain were identified by growth at 37°C in TB medium to an A600 of 0.6. IPTG was added (0.4 μM) and following 2 h growth at 37°C, expression was assessed by determining the absorbance of the cells over the 400–500-nm wavelength range to confirm the presence of induced heme production. Production of the heme domain by the clone pET3aNR1 was further assessed over a 4-h time period using SDS-PAGE and Western blotting as previously described (14). To purify this protein, expressed cells (2 h after IPTG addition) were harvested, resuspended in buffer, and disrupted in a French pressure cell (15,000 p.s.i.). The clarified homogenate was subjected to preparative isoelectric focussing over a pH range of 4–10 using a Bio-Rad IEF Rotofor (Melville, NY). The domain was further purified by FPLC using ion-exchange (Mono-Q) and gel filtration. Purity of this protein, assessed by SDS-PAGE, was estimated at approximately 80% with an A280/A450 of < 0.3. For amino acid sequencing studies, the pETANR1 heme domain was further purified by reversed-phase HPLC as previously described (16).

Sizing of Expresssed Proteins—The size of the expressed proteins obtained from the clones pET5bNR1 and pET3aNR1 were estimated by SDS-PAGE followed by immunoblotting, while the oligomeric structure of the expressed domains were determined by gel filtration on a calibrated Superdex 75 column.

Amino Acid Sequencing—Amino acid sequencing was performed using an Applied Biosystems 473A protein sequencer using Edman chemistry (16).

Spectroscopic Studies—UV-visible spectra of the purified domains were obtained using a Shimadzu (Columbia, MD) UV2101PC spectrometer. Circular dichroism spectra were obtained using a JASCO (Baltimore, MD) J710 spectropolarimeter.

Potentiometric Titrations—Visible potentiometric titrations were performed at 25 °C as previously described (5). Proteins, generally in the concentration of range 3-5 μM heme were reduced using reduced methyl viologen radical cation (20 mM) or oxidized using K3Fe(CN)6 (20 mM) in 50 mM MOPS buffer, pH 7.0, containing 0.1 mM EDTA, in the presence of the following mediators (3-5 μM each mediator): DCPIP (E0, = +317 mV), 1,2-naphthoquinone (E0, = +135 mV), 1,4-naphthoquinone (E0, = +60 mV), methylene blue (E0, = +10 mV), pyocyanin (E0, = -60 mV), indigodisulfonate (E0, = -125 mV), and anthraquinone 2-sulfonate (E0, = -225 mV). Heme reduction was monitored at 423 nm using the isosbestic point at 417 nm to correct for background absorbance changes. Titrations were performed in both reductive and oxidative directions.

RESULTS

Protein Expression and Purification—The expression clones pET5bNR1 and pET3aNR1 code for the entire heme-binding domain of Chlorella NR, as shown in Fig. 1. In addition to the heme-binding domain, pET5bNR1 also encoded approximately 30% of the Mo-pterin-binding domain of NR, while pET3aNR1 was constructed to specifically code for the 92 amino acids that have been proposed to form the minimal heme-binding domain as determined from sequence alignment with the corresponding primary structure of calf cytochrome b6 (13). The amino acid sequences of the clones pET5bNR1 and pET3aNR1 exhibit a high degree of similarity to corresponding segments of nitrate reductase from other sources such as that deduced from cDNA analysis of Arabidopsis thaliana (17). While the expression of pET5bNR1 in E. coli has previously been shown to result in the production of a 35-kDa peptide (14), cells expressing pET3aNR1 as a polypeptide showed the IPTG-inducible synthesis of a 10-kDa subunit as shown in Fig. 2. Cells expressing the pET3aNR1 plasmid expressed a protein that immunologically cross-reacted with anti-(Chlorella) NR antibodies and exhibited a distinctive red coloration indicative of the synthesis of a large quantity of heme. The time course of expression of the 10-kDa recombinant NR heme-binding domain is also shown in Fig. 2.

Both of the expressed proteins could be purified, though to varying degrees, using a combination of ammonium sulfate fractionation, ion exchange, and gel filtration chromatography. The pET5bNR1 clone produced a significant amount of the 35-kDa protein (approximately 3–5% of total protein). Gel filtration of this protein using a Superose 6 column indicated a very high molecular weight for the native species, greater than that of the native enzyme, suggestive of protein aggregation. In contrast, the level of expression produced by the pET3aNR1 clone was greater, corresponding to approximately 10% of cellular protein.

Amino acid sequencing of the N-terminal portion of the for background absorbance changes. Titrations were performed in both reductive and oxidative directions.
HPLC-purified 10-kDa recombinant protein yielded a single sequence corresponding to PAGAKSFTMAEV, in agreement with that predicted from cDNA analysis (14).

Size of Recombinant Proteins—The molecular size of the 10-kDa protein was estimated by gel filtration under nondenaturing conditions and is shown in Fig. 3. The protein eluted from a Superdex 75 column at a $K_m$ of 4.7 corresponding to a protein of approximately 45 kDa, suggesting a tetrameric structure under native conditions. Native Chlorella NR also exists as a homotetramer (18).

Spectroscopic Studies—UV-visible spectra obtained for the purified recombinant 10-kDa heme protein in the oxidized and dithionite-reduced forms are shown in Fig. 4. The oxidized spectrum, typical of a $b_5$-type cytochrome exhibited maxima at 278, 413, 529, and 560 nm. Reduction of the protein, resulted in an increased absorption in the visible region with new maxima at 424, 528, and 557 nm, respectively.

Circular dichroism spectra for the 10-kDa recombinant protein are shown in Fig. 5. In the oxidized state, the protein exhibited positive CD at 377 nm and negative CD at 408 nm, respectively. Reduction of the protein using excess dithionite, resulted in the appearance of positive CD bands at 417 nm and negative CD bands at 427, 544, and 565, respectively. Similar CD spectra, were obtained for both the oxidized and reduced forms of native NR.

Potentiometric Titrations—Oxidation-reduction potentiometric titrations in the presence of dye mediators for both recombinant proteins, obtained from clones pET5bNR1 and pET3aNR1, are shown in Fig. 6. The purified 35-kDa protein yielded a reversible, $n = 1$ redox curve, corresponding to a midpoint potential of $-28$ mV. In contrast, for the 10-kDa protein, the midpoint potential was determined to be $+16$ mV.

FIG. 5. Circular dichroism spectra of the 10-kDa recombinant heme domain. CD spectra of the core heme domain of NR and native Chlorella NR in 50 mM phosphate buffer, pH 7.6, were determined before and after reduction with excess dithionite. The heme concentration for both samples was 6 mM. Solid line, oxidized 10-kDa heme domain; dashed line, reduced 10-kDa heme domain; dotted line, oxidized native Chlorella nitrate reductase; dashed and dotted line, reduced native Chlorella nitrate reductase.

FIG. 6. Potentiometric titration of the core heme domain of two recombinant heme domains of nitrate reductase during potentiometric titration. Proteins (3–5 $\mu$M heme) in 50 mM MOPS buffer, pH 7.0, containing 0.1 mM EDTA, were anaerobically reduced with reduced methyl viologen radical cation (20 mM) (solid symbols) or oxidized with ferricyanide (20 mM) (open symbols) in the presence of various dye mediators. Spectra were recorded following attainment of equilibrium, and the relative absorbance changes were plotted as a function of the applied potential. Redox titrations were determined for the 35-kDa protein (squares) and 10-kDa protein (circles) and compared to the potential obtained for native Chlorella nitrate reductase (right line).

DISCUSSION

Extensive spectral and primary structure studies have demonstrated that assimilatory NADH:nitrate reductase is a member of the superfamily of $b_5$-type cytochrome-containing proteins (17) which comprises, in addition, mitochondrial cytochrome $b_6$, yeast flavocytochrome $b_6$, and hepatic sulfite oxidase. Within the amino acid sequences of these complex metalloproteins, the portions of the protein corresponding to the heme-binding domain have been identified by their extensive sequence similarity to that of cytochrome $b_6$. For assim-
Nitrile reductase heme domain has been localized to the central portion of the sequence comprising the 92 amino acids beginning with residue 539 in the NR sequence (this numbering scheme refers to the sequence obtained for Arabidopsis thaliana as the base sequence (17)). The N-terminal portion (residues 1–538) of the NR sequence has been identified as containing the Mo-pterin prosthetic group, while the remaining, approximately 300 C-terminal residues, comprise the FAD-binding domain. The boundaries of these domains have been verified both by limited proteolysis studies (6,19) and by the expression of individual recombinant proteins containing the heme and flavin domains, respectively (14, 20, 21).

Using recombinant technology, we have been able to synthesize and isolate two heme-binding domains that differ significantly in size, approximately 35 versus 10 kDa, with the former containing approximately 30% of the Mo-pterin binding domain. We have verified the N-terminal amino acid sequence of the small fragment and have shown that it corresponds to that predicted from the cDNA analysis. Gel filtration studies indicate that the 10-kDa heme domain was synthesized in E. coli as an oligomer of four subunits. Since native Chlorella NR is a dimer of dimers (18), this result suggests that the subunit association site is located in the heme-binding domain. This is consistent with limited proteolysis studies where cleavage of native NR, by V8 protease, released the FAD domain but did not disrupt the oligomeric nature of NR (6), indicating the subunit interaction site is not located in the FAD domain. Oligomeric structure of expressed NR has also been seen with the tobacco NR cDNA expressed in Saccharomyces cerevisiae (22). This recombinant protein was synthesized as a dimer without associated Mo-pterin suggesting this prosthetic group does not have a role in subunit interaction.

The UV-visible and CD spectra of the recombinant heme-binding domains, both in the oxidized and reduced states, are very similar to the spectra obtained for the native Chlorella NR. These spectra are sensitive to the nature of the heme electronic environment indicating no significant perturbation of the heme structure in the recombinant proteins. However, in contrast, our thermodynamic studies of these recombinant heme proteins have indicated significant differences between the midpoint potentials obtained for the heme protoporphyrin group in these isolated domains compared with the values obtained for the native protein. We have previously shown (Table 1) that separation of the Mo/heme fragment from the flavin domain of NR using limited proteolysis resulted in no significant change in the heme midpoint potential \( E' = -166 \) mV from that of the native enzyme \( E' = -160 \) mV indicating that none of the amino acid residues comprising the interface between the heme and flavin domains are involved in maintaining the low redox potential of the heme prosthetic group in native NR. However, the heme prosthetic group in the recombinant 35-kDa protein exhibited a midpoint potential of \(-28 \) mV, significantly higher than that of the native enzyme. In addition, this midpoint potential is further elevated to \(+16 \) mV in the 10-kDa recombinant protein. Thus, truncation of the NR heme domain to a size approximately equal to that of cytochrome \( b \), resulted in a comparable heme midpoint potential, indicating that portions of the amino acid sequence located on the N-terminal side of the heme domain significantly influence the midpoint potential of this prosthetic group. This potential modulation of the heme midpoint potential by the addition of amino acid residues on the N-terminal portion of the heme domain appears to be unique to assimilatory nitrate reductase since a similar expression system has been developed for the heme domain of the closely related protein, flavocytochrome \( b \) (23). For this reason, the expression system which repressed heme domain, which represents the N-terminal portion of the native enzyme, exhibits a midpoint potential comparable to that of the heme in the holoenzyme.

These results provide evidence that the midpoint potential of the heme prosthetic group can be modulated by N-terminal peptide sequences. Consequently, it may be possible to modify heme potential of NR and thus alter the rate of electron transfer. This ultimately could modulate NR activity since the transfer of electrons from the heme group to the Mo-pterin appears to be rate-limiting in NR catalysis (4).

Acknowledgments—We are grateful to Dr. Peter Neame for the amino acid sequencing and Dr. Christopher Kay for some of the preliminary spectroscopic and thermodynamic studies of these proteins.

REFERENCES

<table>
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<th>Table I</th>
<th>Oxidation-reduction midpoint potentials for the heme prosthetic group of nitrate reductase and related proteins</th>
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<tr>
<td>Protein</td>
<td>( E' ) Reference</td>
</tr>
<tr>
<td>Nitrate reductase (Candida nitratophila)</td>
<td>−174</td>
</tr>
<tr>
<td>W-substituted NR (C. vulgaris)</td>
<td>−168</td>
</tr>
<tr>
<td>Heme/Mo-pterin NR (minus flavin) (C. vulgaris)</td>
<td>−166</td>
</tr>
<tr>
<td>Nitrate reductase (C. vulgaris)</td>
<td>−160</td>
</tr>
<tr>
<td>Nitrate reductase (Spinach)</td>
<td>−127</td>
</tr>
<tr>
<td>Nitrate reductase (Monophorium)</td>
<td>−73</td>
</tr>
<tr>
<td>Recombinant 35-kDa NR heme domain</td>
<td>−28</td>
</tr>
<tr>
<td>Recombinant 10-kDa NR heme domain</td>
<td>+16</td>
</tr>
<tr>
<td>Recombinant flavocytochrome ( b_2 ) (minus flavin)</td>
<td>−31</td>
</tr>
<tr>
<td>Flavocytochrome ( b_2 ) (minus flavin) (S. cerevisiae)</td>
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</tr>
<tr>
<td>Flavocytochrome ( b_2 ) (S. cerevisiae)</td>
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<tr>
<td>Microsomal Cytochrome ( b_2^e )</td>
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</tr>
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<td>Sulfite oxidase</td>
<td>+77</td>
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\(^{a}\) From beef liver. \(^{b}\) From chicken liver.