Spectroscopic and Kinetic Characterization of the Recombinant Wild-type and C242S Mutant of the Cytochrome b Reductase Fragment of Nitrate Reductase*

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Spectroscopic and kinetic studies comparing the behavior of the recombinant cytochrome b reductase fragment of corn leaf nitrate reductase and a mutant in which cysteine 242 is replaced with a serine residue (C242S) have been carried out. The visible and circular dichroism spectra of the wild-type and mutant protein are virtually identical and compare well with those reported for nitrate reductases from other sources. The reduced wild-type protein forms a charge-transfer complex with NADH that has an absorption envelope that extends into the near infrared, with a maximum around 800 nm. The C242S mutant forms a similar charge-transfer complex with NADH but to a lesser extent than the wild-type. The reduction potential of the flavin for the wild-type protein is −287 mV, and that for the mutant is −279 mV. The rate of reduction by NADH of the C242S mutant is 7-fold slower than that for the wild-type protein, and the Kd for the mutant is larger by a factor of 2. These results indicate that the cysteine 242 residue plays a role principally in facilitating electron transfer from NADH to the flavin rather than in binding of NADH to the enzyme.

Nitrate reductase (EC 1.6.6.1-3) catalyzes pyridine nucleotide-dependent reduction of nitrate to nitrite as the first step in nitrogen assimilation in algae, fungi, and higher plants. The enzyme is a multicentered redox enzyme containing FAD, a b-type cytochrome, and a molybdenum center (1–3). Each prosthetic group appears to be found in structurally independent fragments of the two identical ~100-kDa subunits of nitrate reductase. Catalytically active fragments have been demonstrated and even purified subsequent to mild proteolysis (2, 4). Genes of numerous nitrate reductases have been cloned, and it is evident that the apparent prosthetic group binding regions of nitrate reductase are laid out in a linear fashion in the amino acid sequence on the basis of sequence homology to enzymes of simpler functionality (2, 3, 5). For example, the N-terminal fragment of nitrate reductase has high similarity to the molybdenum binding region of mammalian sulfite oxidase (6, 7). An internal fragment of nitrate reductase has sequence similarity to the soluble domain of mammalian and higher plant cytochrome b5, and the C-terminal fragment of nitrate reductase is highly similar in sequence to the oxidation-reduction partner of cytochrome b5, namely, NADH-cytochrome b5 reductase (2, 3, 5). The similarity between the cytochrome b reductase fragment of nitrate reductase and cytochrome b5 reductase is especially extensive (8).

The structural independence of the several cofactor-binding fragments of nitrate reductase is further supported by the independent expression of two of these, the FAD- and heme-containing fragments, in Escherichia coli as stable and redox-active polypeptides (8–10). The x-ray crystallographic structure of the FAD-containing cytochrome b reductase fragment of nitrate reductase has recently been reported (11), and a potential new role for one of the enzyme’s key cysteine residues has been identified by site-directed mutagenesis of the recombinant fragment (12). The present work is an extension of these studies of the recombinant cytochrome b reductase fragment of nitrate reductase focusing on its oxidation-reduction chemistry and rapid reaction kinetic behavior.

The three-dimensional model of the cytochrome b reductase fragment of nitrate reductase has established its FAD-binding region as a member of the structurally related, but a functionally diverse group of flavin reductases known as the “ferredoxin NADP⁺ reductase family” (11). The structure of ferredoxin NADP⁺ reductase was the first of this family to be elucidated (13). The sequence similarity of the cytochrome b reductase fragment of nitrate reductase and ferredoxin NADP⁺ reductase had been recognized before the structure was derived, but only a few key residues are strictly conserved (13, 14). Other members of this group include phthalate dioxygenase reductase, an FMN-containing NADH-dependent bacterial enzyme (15–17) and cytochrome b5 reductase (18). Several of these enzymes are spectroscopically and kinetically well characterized (19–22).

For many years, it has been recognized that nitrate reductase has a cysteine that can be protected by pyridine nucleotides (1–3), and similar active site cysteines have been identified in other members of the ferredoxin NADP⁺ reductase family of flavoenzymes (16, 19, 21, 23). Chemical modification of this cysteine by sulfhydryl reagents significantly reduces activity. There are only two invariant cysteine residues among nitrate reductases from various organisms, one in the molybdenum-containing fragment and one in the cytochrome b reductase fragment (5, 7). Since the cysteine in the molybdenum-containing region has been suggested to be involved in binding the cofactor (7), the cysteine in the FAD-binding region is the obvious candidate as the catalytically essential one, especially considering the similarity of this region to the ferredoxin NADP⁺ reductase family of enzymes (13, 14). To determine if the invariant cysteine of the recombinant cytochrome b reductase fragment of nitrate reductase is the reactive one, each of the five cysteine residues of this fragment have been changed to another amino acid, and it is found that only by converting the cysteine 242 to a serine residue (C242S) is the ferricyanide reductase activity rendered insensitive to p-hydroxymercu-
ribenzoate (12). Subsequently the structural model for this fragment of nitrate reductase has revealed that cysteine 242 extends into the active site crevice of the recombinant protein (11). The steady-state kinetics of the wild-type and C242S mutant of the cytochrome b reductase are found to be quite different, with the mutant having a $k_{cat}$ of about one-sixth of the wild-type; $K_m$ values for NADH and ferriyanide are essentially unchanged (12). These results suggest that cysteine 242 is important for reduction of the protein-bound flavin. Most recently, the structural model for the cytochrome b reductase fragment has been extended to include an analysis of the C242S mutant, which indicates the side chain of the substituted serine forms a hydrogen bond with the main chain oxygen atom of glycine 147 that is not present in the wild-type protein. This interaction causes a local conformational change that results in a large void in the active site (24). Here we report a more detailed comparison of the spectroscopic and kinetic properties of the wild-type and C242S mutant of the recombinant cytochrome b reductase fragment in order to gain a better understanding of the role of cysteine 242 in flavin reduction by NADH.

MATERIALS AND METHODS

The recombinant wild-type and C242S mutant cytochrome b reductase fragments of corn leaf nitrate reductase were obtained as described previously (8, 12). All experiments were done in 0.1 M MOPS, 0.1 M KCl, pH 7.0, containing 0.1 mM EDTA, unless otherwise stated. Ultraviolet/visible spectra were recorded using a Hewlett Packard 8452A single-beam diode array spectrophotometer; visible/near-infrared spectra were recorded with an On-Line Instrument Systems (OLIS) modernized Cary-14. Circular dichroism (CD) spectroscopy was carried out using an AVIV 40DS spectrophotopolarimeter. Reductive titrations were carried out using anaerobic glassware described previously (25). Samples were made anaerobic by alternately evacuating and flushing with oxygen-free argon on an anaerobic train and then titrated with either sodium dithionite or NADH solution in 0.1 M MOPS, 0.1 M KCl, pH 7.0, containing 0.1 mM EDTA that had previously been made anaerobic by bubbling with oxygen-free argon. The reduction potentials of the wild-type and mutant enzymes were determined as described by Massey (26), using safranine T as the standard ($\Delta E^\circ = -289 mV$) (27). Rapid mixing experiments were carried out using a Kinetic Instruments Inc. stopped-flow apparatus interfaced with an OLIS model 3920Z data collection system. Samples were placed in a tonometer equipped with a three-way stopcock valve with a male Luer connector and made anaerobic by alternately evacuating and flushing with oxygen-free argon. The tonometer was mounted on the stopped-flow apparatus, and samples were then rapidly mixed with anaerobic NADH solutions. The kinetic transients obtained after mixing were monitored as transmittance voltages collected by a high speed A/D converter and converted to absorbance changes, and the rate constants were determined by OLIS Inc. software.

Sodium dithionite was obtained from Virginia Chemicals Co. Safranine T was a gift from the laboratory of Dr. Vincent Massey, University of Michigan. NADH, NAD$^+$, and 3-aminopyridine adenine dinucleotide (AAD$^-$) were obtained from Sigma. All other chemicals were of reagent grade and were used without additional purification.

RESULTS AND DISCUSSION

Visible and CD Spectra—The visible and CD spectra of the wild-type and C242S mutant cytochrome b reductase fragment of nitrate reductase are very similar (Fig. 1). The absorbance spectra for both wild-type and C242S-oxidized proteins exhibit absorbance maxima at 392 and 460 nm and a shoulder at 482 nm (Fig. 1, A and C), which are very similar to those of human cytochrome b$_6$ reductase (8, 12, 21). When the enzyme is reduced with sodium dithionite in the presence of NAD$^+$, a long wavelength absorption band appears that is attributed to the formation of a charge-transfer complex between reduced flavin and NAD$^+$. This long wavelength absorption, which is also

$^1$ The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; AAD$^-$, 3-aminopyridine adenine dinucleotide.
Nitrate Reductase's Cytochrome b Reductase

observed in the wild-type and C242S mutant upon reduction by NADH, extends well into the near-IR region, with a maximum at around 800 nm (Fig. 1, A and C, inset). A similar charge-transfer complex between NAD\textsuperscript{+} and reduced cytochrome b\textsubscript{5} reductase has been observed (21). Spectroscopic evidence has also been presented for a charge-transfer complex between NAD\textsuperscript{+} and reduced holonitrate reductase from Chlorella vulgaris (28). Iyanagi et al. (29) have suggested that the reduced flavin-NAD\textsuperscript{+} complex of cytochrome b\textsubscript{5} reductase serves to protect the reduced enzyme against rapid oxidation by molecular oxygen. A similar role for the reduced flavin-NAD\textsuperscript{+} complex of the recombinant cytochrome b reductase fragment and its parent enzyme, nitrate reductase, can also be suggested since reduced enzyme reacts only slowly with oxygen. While the absorbance spectra of the charge-transfer complex for both wild-type and mutant enzymes are similar in appearance, the absorption of the complex formed by the wild-type is approximately 30% greater than that of the mutant. This appears to be due not to the less extensive formation of the NAD\textsuperscript{+} complex in the C242S mutant but rather a less effective interaction between FAD\textsubscript{H} and NAD\textsuperscript{+} within the fully formed complex.

The CD spectra of the wild-type cytochrome b reductase fragment and C242S mutant are also similar in each of the three states examined (oxidized, reduced, and reduced plus NAD\textsuperscript{+}) (Fig. 1, B and D). However, as noted with the absorbance spectra of the charge-transfer complexes, the CD spectra of the reduced forms of wild-type and C242S mutant of cytochrome b reductase in the presence of NAD\textsuperscript{+} have slight differences with the mutant form having lower intensity (Fig. 1, B and D). Both the absorption and CD spectra of the oxidized forms of wild-type and C242S cytochrome b reductase fragment also compare well with those reported for the proteolytic fragment containing the FAD domain of nitrate reductase from C. vulgaris (22, 30). The present results simultaneously confirm the integrity of the wild-type recombinant and C242S proteins and the assignment of the CD of nitrate reductase to its flavin site (30). Fluorescence of the FAD centers in both wild-type and mutant proteins were examined, and there are no noticeable differences. The emission spectra have a maximum at approximately 525 nm (\(\lambda_{\text{ex}} = 460\) nm) (data not shown).

NAD\textsuperscript{+} and the NAD\textsuperscript{+} analog AAD\textsuperscript{+} also bind to the oxidized forms of the wild-type and C242S mutant as shown by difference spectra (Fig. 2, A and B). A previous study of the interaction of the Neurospora crassa NADPH nitrate reductase with AAD\textsuperscript{+} and AAD\textsuperscript{+} phosphate (an NAD\textsuperscript{+} analog derived from AAD\textsuperscript{+}) demonstrated that the analogs were competitive inhibitors of NADPH (31). In the case of both the wild-type and mutant cytochrome b reductase fragment of the corn enzyme, AAD\textsuperscript{+} perturbs the absorbance spectrum of oxidized enzyme to a greater extent than does NAD\textsuperscript{+}, as is seen by a comparison between the difference spectra; the effect is particularly pronounced in the 300–400 nm region. The contribution of free AAD to the original spectrum was subtracted prior to obtaining the difference spectrum. Mutation of cysteine 242 to serine has little effect on the NAD\textsuperscript{+}-induced difference spectrum, while there is a rather more substantial effect on the AAD\textsuperscript{+} difference spectra, at least below 400 nm. This presumably reflects differences in the flavin environment between the two forms. The x-ray crystallographic derived model of the C242S mutant shows that while the side-chain of cysteine 242 in the wild-type is free to interact with NAD\textsuperscript{+}, the serine hydroxyl of the mutant is hydrogen-bonded to the main chain oxygen atom of glycine 147, resulting in a local conformational change (24).

Reductive and Potentiometric Titrations—Reductive titrations of the wild-type and mutant cytochrome b reductase with NADH yielded similar spectral sets (Fig. 3). In both cases, a simultaneous increase in absorbance in the long wavelength region is observed with decrease in absorbance in the 350–500 nm region, suggesting the formation of a charge-transfer complex between reduced enzyme and NAD\textsuperscript{+} subsequent to enzyme reduction. A comparison of the spectra obtained with enzyme reduced with sodium dithionite (Fig. 1, A and C), and enzyme reduced with NADH indicates that NADH does not reduce the enzyme fully. Partial reduction of either wild-type or mutant enzyme with sodium dithionite did not resolve the formation of any semiquinone species, which is different than the case of cytochrome b\textsubscript{5} reductase where partially reduced enzyme yielded a semiquinone species still in the charge-transfer complex with NAD\textsuperscript{+} (29).

Potentiometric titrations have been performed with both wild-type and mutant enzymes by the method described by Massey (26). The data (Fig. 4) have been plotted as the logarithm of oxidized/reduced concentrations of the unknown versus the logarithm of the oxidized/reduced concentrations of the standard dye (32), in this case, safranine T (\(\Delta E^0 = -289 \text{ mV}\)). The reduction potential determined for the FAD/FADH\textsubscript{2} couple for the wild-type enzyme thus obtained is \(-287 \text{ mV}\). Mutation of the cysteine 242 to serine increases the reduction potential only very modestly to \(-279 \text{ mV}\). In the presence of NAD\textsuperscript{+}, the

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with sodium dithionite, and reduced in the presence of excess NAD\textsuperscript{+} (with same designations as in A). The inset shows the absorbance spectrum of the charge-transfer complex in the visible and near-IR region. D, CD spectra of the C242S mutant in the oxidized form (C---C), reduced with sodium dithionite (---), and reduced in the presence of excess NAD\textsuperscript{+} (-----).
potential of the wild-type enzyme increases by 22 mV \((E = -265 \text{ mV})\). The C242S mutant also exhibits an increase in potential in the presence of NAD\(^+\), but the increase is only approximately half of that observed in the case of the wild-type \((E = -268 \text{ mV})\). Overall, these results are consistent with the conformation of the active site in the C242S mutant being minimally altered relative to the wild-type \((24)\), which is reflected in the rather small change in the prosthetic group's reduction potential. On the other hand, the smaller change in reduction potential of the FAD upon binding NAD\(^+\) implies that a less extensive charge-transfer interaction occurs in the mutant as compared with wild-type, which correlates well with the less intense long wavelength absorbance and CD spectrum of the mutant when complexed with NAD\(^+\) as compared with wild-type (Fig. 1).

Kinetic Analysis—The reaction of oxidized enzyme with NADH was examined by rapidly mixing the enzyme with different concentrations of NADH under anaerobic conditions (Fig. 5). The reduction of the flavin was monitored at 460 nm, and the formation of the charge-transfer complex was monitored at 800 nm. Initially, these analyses were carried at 25 °C; however, data could be collected on only the mutant at this temperature since the reaction of the wild-type with NADH was completed within the dead-time of the instrument at 25 °C. Subsequently, the reaction was studied at 10 °C where both cytochrome \(b\) reductase forms reacted with NADH more slowly. The kinetic transients at 460 and 800 nm are monophasic for both the wild-type and the mutant enzymes, and the rate of reduction of the flavin is comparable with the rate of formation of the charge-transfer complex. Double-reciprocal plots were used for determining \(k_{\text{red}}\) and \(K_q\) (Fig. 6). At 10 °C, the kinetic parameters for wild-type cytochrome \(b\) reductase were determined to be a \(K_q\) of 3 \(\mu\)M and \(k_{\text{red}}\) of 474 s\(^{-1}\) and for the C242S mutant a \(K_q\) of 6 \(\mu\)M and \(k_{\text{red}}\) of 68 s\(^{-1}\). At 25 °C, the C242S mutant cytochrome \(b\) reductase had \(k_{\text{red}}\) of 193 s\(^{-1}\). This rate of reduction of the cytochrome \(b\) reductase C242S mutant compares well with the steady-state kinetic rate for the overall reaction of NADH and ferricyanide, which was previously found to be 230 s\(^{-1}\) \((12)\). When the ratio of the rate of reduction of cytochrome \(b\) reductase C242S mutant by NADH at the two temperatures \((\text{i.e.} \ 193 \text{ s}^{-1}/68 \text{ s}^{-1} = 2.84)\) is used to estimate the rate of reduction by NADH of the wild-type at the higher temperature, a value of \(-1350 \text{ s}^{-1}\) is found for the wild-type reduction rate by NADH at the higher temperature, and this compares well with the previously determined steady-state \(k_q\) of 1330 s\(^{-1}\) found for the wild-type at 25 °C for the reduction of ferricyanide by NADH \((12)\).

**CONCLUSION**

Analyses of UV/visible absorbance spectra have indicated small differences between the wild-type and C242S mutant cytochrome \(b\) reductase fragment of nitrate reductase. In the presence of sodium dithionite-reduced enzyme, NAD\(^+\) forms a charge-transfer complex that has a long wavelength absorbance with a maximum at 800 nm. Other pyridine-nucleotide dependent flavoproteins that are members of the ferredoxin

**Fig. 3. Reductive titrations with NADH.** A, titration of the wild-type nitrate reductase's cytochrome \(b\) reductase fragment with NADH under anaerobic conditions. B, titration of the C242S mutant with NADH under anaerobic conditions.

**Fig. 4. Potentiometric titrations of nitrate reductase's cytochrome \(b\) reductase fragment.** The concentration of enzyme used ranged between 15 and 30 \(\mu\)M. The enzyme was incubated under anaerobic conditions with an equal concentration of safranine T, 1.6 \(\mu\)M methyl viologen and with 240 \(\mu\)M xanthine, and 50 \(\mu\)M xanthine oxidase. Enzyme reduction was monitored at 410 nm, an isosbestic point in the reduction of safranine T, and the dye was monitored either at 518 or 540 nm. A, data obtained for the wild-type enzyme in the absence of NAD\(^+\) (closed circles) and in the presence of NAD\(^+\) (closed squares). B, data obtained for the C242S mutant in the absence of NAD\(^+\) (open circles) and in the presence of NAD\(^+\) (open squares).
The NADP⁺ reductase family of flavoproteins and holonitrate reductase are known to form similar complexes (19, 21, 28). The mutant enzyme forms a similar complex but to a lesser extent than that of the wild-type recombinant fragment of nitrate reductase. NAD⁺ also perturbs the spectrum of oxidized enzyme as is seen in the difference spectrum of oxidized enzyme and enzyme in the presence of NAD⁺ (Fig. 2A). The NAD⁺ analog AAD⁺ also perturbs the spectrum of oxidized enzyme (Fig. 2B). The difference spectrum observed with the C242S mutant is somewhat different than that obtained for the wild-type cytochrome b reductase fragment, indicating changes in the flavin environment that affect binding of inhibitor versus product. The potential determined for the FAD/FADH₂ couple in the wild-type recombinant corn leaf nitrate reductase cytochrome b reductase fragment (E = -287 mV, Table I) is within the range of the values determined for the holonitrate reductases from other sources (30, 33). In the presence of NAD⁺, the reduction potential of the FAD of the recombinant nitrate reductase cytochrome b reductase fragment increases by 22 mV (Table I), a considerably smaller shift than in the case of the proteolytically generated FAD-containing fragment of holonitrate reductase from C. vulgaris, where the potential was observed to increase by 60 mV (2). It should be noted that the midpoint potentials of holonitrate reductase are highly influenced by pH with the FAD reduction potential having an approximate decrease of 30 mV/pH unit in the range of pH 6–9 (30). The major difference between the wild-type recombinant cytochrome b reductase fragment and its C242S mutant, however, is observed in their kinetic behavior (Table I). The rate of reduction of the wild-type fragment by NADH is found to be about 7-fold greater than that of the mutant and Kₐ for NADH about half of that for the mutant. The kₕ/kₐ for the wild-type fragment by NADH is 14-fold greater than that of the C242S mutant (Table I). The Kₐ for NADH differing only by a factor of two between the wild-type and mutant probably means that cysteine 242 contributes no more than 0.5 kcal/mol to the binding of NADH. This indicates that cysteine 242 plays more of a role in reducing the activation barrier to flavin reduction than in binding of substrate. The effect on the activation energy to flavin reduction is about 1 kcal/mol, making the sum approximately 1.5 kcal/mol, which is low but within the range for a hydrogen bonding interaction. This seems consistent with the fact that in the wild-type enzyme, the cysteine is free to interact in such a manner with the substrate but in the mutant the serine is already hydrogen bonded to glycine 147 and is unable to interact in a similar fashion with the substrate. The reduced half-reaction kinetic results are in agreement with earlier steady-state kinetic work that indicated that there was about a 6-fold decrease in kₕ on substituting a serine for cysteine 242 in a NADH:ferricyanide reductase activity assay (12, Table I). Thus, flavin reduction by NADH appears to be the rate-limiting step in the overall catalysis of the recombinant cytochrome b reductase fragment of nitrate reductase when ferricyanide is the electron acceptor. A similar conclusion that FAD reduction.
by NADH limited catalysis has been reached for cytochrome b subunit reductase (21). Finally, based on the recent structural models for both the wild-type and C242S mutant of corn leaf nitrate reductase's recombinant cytochrome b reductase fragment (11, 24), it can be concluded that the thiol side chain of cysteine 242 may play a role in positioning NADH for efficient electron transfer but does not participate in catalysis directly. Thus, while cysteine 242 promotes catalytic efficiency of the cytochrome b reductase fragment (and presumably of holonitrate reductase where it is an invariant residue in over 30 examples of nitrate reductase sequences from a wide variety of species of higher plants, algae, and fungi), it is not essential for NADH reduction of the enzyme flavin. Hence, the evolutionary pressure to retain the invariant cysteine of the FAD reduction active site of nitrate reductase and most of the other members of the ferredoxin NADP+ reductase family of flavoprotein reductases is not because a mutation results in an inactive form but rather in a catalytically inefficient one.

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