**Spectroscopic and Kinetic Characterization of the Recombinant Cytochrome c Reductase Fragment of Nitrate Reductase**

**IDENTIFICATION OF THE RATE-LIMITING CATALYTIC STEP**

(Received for publication, August 9, 1996, and in revised form, November 6, 1996)

Kapila Ratnam‡, Naomasa Shiraishi§, Wilbur H. Campbell§, and Russ Hille‡‡

From the ‡Department of Medical Biochemistry, Ohio State University, Columbus, Ohio 43210 and §Phytotechnology Research Center and Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931

The recombinant NADH-cytochrome c reductase fragment of spinach NADH-nitrate reductase (EC 1.6.6.1), consisting of the contiguous heme-containing cytochrome b domain and flavin-containing NADH-cytochrome b reductase fragment, has been characterized spectroscopically and kinetically. Reductive titration with sodium dithionite indicates heme reduction takes place prior to flavin reduction, which correlates well with the reduction potentials for enzyme-bound heme (15 mV) and FAD (~280 mV). Reductive titration with NADH also indicates that the reduced enzyme forms a charge-transfer complex with NADH. The circular dichroism spectrum of the oxidized fragment is primarily due to the flavin, whereas the ferrous heme dominates the circular dichroism spectrum of reduced enzyme. Three kinetic phases are observed in the course of the reaction of the enzyme with NADH, each with a distinct spectral signature. The fast phase represents flavin reduction, concomitant with the formation of a charge-transfer complex between reduced flavin and NADH, and exhibits hyperbolic dependence on NADH concentration with a K_d of 3 μM and a limiting rate constant of 560 s^{-1}. Electron transfer from reduced flavin to heme with a rate constant of 12 s^{-1} is the intermediate phase, which is rate-limited by breakdown of the charge-transfer complex between NADH and reduced flavon. The slow phase is dismutation of a pair of molecules of two-electron reduced enzyme (generated at the end of the second phase of the reaction) to give one molecule each of one- and three-electron reduced enzyme, with a second order rate constant of 2 × 10^6 M^{-1}s^{-1}. In the presence of excess NADH, this dismutation reaction is followed by the rapid reaction of the one-electron reduced enzyme with a second equivalent of NADH to generate fully reduced enzyme. On the basis of this work, it appears that dissociation of NADH from the reduced flavin site rate limits electron transfer to the cytochrome and likely represents the overall rate-limiting step of catalysis.

Nitrate reductase (EC 1.6.6.1–3) catalyzes the pyridine nucleotide-dependent reduction of nitrate to nitrite as the first and rate-limiting step in nitrogen assimilation in algae, fungi, and higher plants (1–3). The enzyme is a complex metalloflavoenzyme of molecular weight 200 kDa containing an FAD, a b-type cytochrome, and a molybdenopterin center in each of its two identical subunits (1–3). In the course of the catalytic sequence, reducing equivalents (in the form of NADH) enter the enzyme at the flavin site and are subsequently transferred via the heme to the molybdenum center, where the chemistry associated with nitrate reduction takes place. The redox-active centers are present in structurally independent domains, which are laid out in a linear fashion in the amino acid sequence and which exhibit homology to other enzymes containing similar cofactors (2–4). The N-terminal molybdenum-containing fragment of nitrate reductase possesses sequence homology to the molybdenum binding region of sulfite oxidase (5, 6), the central heme domain to the soluble domain of mammalian and higher plant cytochrome b5 (3) and the C-terminal fragment of nitrate reductase (containing only the FAD) to the physiological partner of cytochrome b5, namely, NADH-cytochrome b5 reductase, as well as other members of the ferredoxin-NADP⁺ reductase family (2–4). The FAD-containing recombinant cytochrome b reductase fragment of corn leaf nitrate reductase has been characterized recently, and the role of cysteine 242 (invariant among nitrate reductases) examined (7, 8). An x-ray crystal structure for this fragment is now available and has established that the protein is a member of the structurally related but functionally diverse group of flavin reductases that comprise the ferredoxin-NADP⁺ reductase family (9).

The central heme domain is connected to the N- and C-terminal domains through hinge regions that are susceptible to proteolytic cleavage. Native nitrate reductase can be easily cleaved into stable fragments that retain partial catalytic activities characteristic of their prosthetic groups (10). The structural independence of the cofactor-binding domains of nitrate reductase is further supported by the ability to independently express the FAD- and heme-containing fragments as stable and redox-active polypeptides (11–13). The recombinant FAD-containing cytochrome b reductase fragment has NADH-ferri-cyano activity and an absorbance spectrum similar to mammalian cytochrome b5 reductase (11). The heme domain, when reduced by sodium dithionite, is able to pass its electron on to cytochrome c (12), and when the heme domain and cytochrome b reductase fragment are expressed as a single polypeptide, the protein exhibits NADH-cytochrome c reductase activity (13). The absorbance spectrum of this cytochrome c reductase fragment of nitrate reductase is virtually indistinguishable from that of native nitrate reductase, with the cytochrome b dominating the spectrum of both oxidized and reduced enzyme. Although a three-dimensional structure for this fragment is as yet unavailable, docking studies have been performed using the structures for the cytochrome b reductase fragment and bovine...
cytochrome \(b_2\), which exhibits 47% amino acid residue identity and 84% sequence homology with the cytochrome \(b\) domain of nitrate reductase (14). The resulting model suggests that the propionate group on the heme comes in close proximity to the C-8 methyl group of the FAD in order for electron transfer from FAD to heme to occur, in a manner similar to flavocytochrome \(b_2\) (15).

Here we report the characterization of the spectroscopic and kinetic properties of the recombinant cytochrome \(c\) reductase fragment of spinach nitrate reductase. Our results indicate that in the course of turnover, electron transfer from the flavin to the heme does not occur until NAD\(^+\) has dissociated from the flavin and that this is likely to be the rate-limiting step of catalysis.

**MATERIALS AND METHODS**

Spinach leaf NADH-nitrate reductase cDNA clone pSPNR117, which contains a 2.3-kilobase insert, was used to prepare the fragment coding for spinach CeR\(^-\) (16; GenBank\textsuperscript{TM} Accession No. U089029). The pSPNR117 clone was partially digested with XhoI to obtain a 1.6-kilobase fragment which was subcloned into Bluescript at the BamHI site. When this subclone was digested with EcoRI, the fragment obtained was subcloned into the unique EcoRI restriction site of the Pichia expression vector pHIL-D2, which is a commercially available vector designed for intracellular expression of proteins (Invitrogen). This construct was used for CeR expression in Pichia pastoris strain his4 GS115, per the instruction manual provided by the manufacturer. The CeR containing pHIL-D2 plasmid was linearized by NotI digestion and transformed into Pichia using the spheroplast transformation method (17). A transformant with slow growth on methanol was selected, which indicated that the CeR coding fragment was most likely integrated in the \(aox1\) gene locus of Pichia. Thus, the CeR expressing transformant is of the type called mut\(^+\), for slow methanol utilization, since the alcohol oxidase I of Pichia is knocked out and only alcohol oxidase II remains active.

The CeR expressing Pichia transformant was grown at 30 °C in 50 ml of BMGY medium for 2 days and then used to inoculate 1 liter of BMGY medium in a 2.4-liter flask, which was grown in a shaking incubator (280 rpm) at 30 °C. After 2 days of incubation, the cells were harvested by centrifugation and resuspended in 1 liter of BMM medium. The CeR expressing Pichia transformant was grown with the addition of 10 ml of 100% methanol every 24 h. After 5 days of growth in methanol-containing medium, the cells were harvested by centrifugation and resuspended in 500 ml of phosphate extraction buffer (50 mM NaPO\(_4\), pH 7.3, 1 mM EDTA). Cells were disrupted using glass beads in a bead-beater extraction device (BioSpec Products). The extract was centrifuged (18,000 \(\times\) g, 20 min at 4 °C), and a 30–50% ammonium sulfate precipitate was prepared which was suspended in phosphate extraction buffer. The ammonium sulfate fraction was applied to blue Sepharose, and after washing with phosphate extraction buffer, the CeR was eluted with NADH as described previously (18). Samples were made anaerobic and then titrated with solutions of either sodium dithionite or NADH in 0.1 M MOPS, pH 7.0, containing 1 mM EDTA using a DIAFLO ultrafiltration stirred cell (Amicon). Purity of the CeR preparations was determined by denaturing polyacrylamide gel electrophoresis as described previously (7).

Protein sequencing of the purified CeR revealed that the second available AUG in the spinach nitrate reductase coding sequence in the Pichia vector construct was where translation had begun. Thus, the CeR fragment expressed here contains spinach nitrate reductase residues 253–926 with a predicted \(M_r = 41,475\). Careful size analysis of the denaturing polyacrylamide gel of purified CeR indicated that the recombinant protein is 41.7 kDa, which confirmed the predicted size. More details of the preparation and properties of the Pichia expressed CeR will be published elsewhere.

All experiments with the recombinant CeR were performed in 0.1 M MOPS, 0.1 N KCl, pH 7.0, containing 0.1 mM EDTA, unless otherwise stated. Ultraviolet/visible spectroscopy was carried out using a Hewlett-Packard 8452A diode array spectrophotometer, and circular dichroism (CD) spectroscopy was carried out on an Aviv 40DS spectrophotopolarimeter. Rapid mixing experiments were performed using a Kinetic Instruments Inc. stopped-flow apparatus interfaced to an On-Line Instrument Systems (OLIS) model 3920Z data collection system. Enzyme samples, in a tonometer equipped with a three-way stopcock valve with a male Luer connector, were made anaerobic by alternating evacuation and flushing with oxygen-free argon on an anaerobic train. The tonometer was mounted on the stopped-flow apparatus and the enzyme rapidly mixed with anaerobic solutions of NADH. Kinetic transients obtained were analyzed and the rate constants determined using OLIS software.

EPR spectra were recorded at 150 K using a Bruker Instruments ER 300 spectrometer equipped with an ER 035 m gaussmeter and a Hewlett-Packard 5352B microwave frequency counter. EPR samples were collected using a rapid quench apparatus that permitted the collection of liquid samples directly into EPR tubes, which were then frozen immediately using a dry ice/acetone bath.

Reductive titrations were carried out in anaerobic glassware as described previously (18). Samples were made anaerobic and then titrated with solutions of either sodium dithionite or NADH in 0.1 M MOPS, 0.1 N KCl, pH 7.0, containing 0.1 mM EDTA that had previously been made anaerobic by bubbling with oxygen-free argon. The reduction potential of the cytochrome \(b\) cofactor was determined as described by Massey (19) using 5-hydroxy-1,4-naphthoquinone (\(\Delta E^\prime\) = −3 mV) (20).

NADH and 5-hydroxy-1,4-naphthoquinone were obtained from Sigma, and sodium dithionite was obtained from Virginia Chemical Co. All other chemicals were of reagent grade and used without additional purification.

**RESULTS AND DISCUSSION**

Visible and CD Spectra—The visible absorption spectrum (Fig. 1A) of the oxidized cytochrome \(c\) reductase fragment of
spinach nitrate reductase is virtually identical to that of the holoenzyme from a variety of sources. The predominant contribution to the spectrum is that of the cytochrome b cofactor with the strongest absorption peak being the Soret band at 414 nm and two broad bands in the region between 500 and 600 nm; the FAD of the protein contributes principally at ~460 nm. When the enzyme is reduced with sodium dithionite, the Soret band shifts to 424 nm and new bands appear at 528 nm and 556 nm; there is a loss of the shoulder at 480 nm due to the bleaching of the FAD upon reduction.

The CD spectrum (Fig. 1B, broken line) of oxidized cytochrome c reductase is mainly due to the flavin and is very similar to that observed for the corn cytochrome b reductase fragment (8). The contribution from the heme domain is primarily in the region between 550 and 600 nm as a small negative band. On the other hand, the CD spectrum of the reduced enzyme (Fig. 1B, solid line) is dominated by the ferrous heme, and the contribution from the flavin hydroquinone, which absorbs weakly above 400 nm, is negligible. The CD spectrum of the reduced enzyme is similar to that observed for the heme domain of the enzyme from Chlorella vulgaris obtained by limited proteolysis (21).

Reductive and Potentiometric Titrations—Equilibrium reductive titrations with dithionite (Fig. 2A) and NADH (Fig. 2B) have been carried out. At pH 7.0, the cytochrome b is reduced earlier in the course of the titration than is FAD, as is seen by a plot of the fractional absorption change at 460 nm (predominantly due to reduction of the flavin) versus 556 nm (due to reduction of the heme) (Fig. 2A, inset). This behavior is expected in light of the significantly higher reduction potential of the heme relative to the flavin (see below). Similarly, with NADH as the reductant, heme reduction occurs earlier in the course of the titration than does FAD reduction (despite it being known that the flavin site is the site of initial reduction by NADH). During the titration with NADH, concomitant with the eventual onset of flavin reduction, there is a net increase in long wavelength absorption (Fig. 2B, inset) indicating the formation of a charge-transfer complex between reduced flavin and NADH+ comparable to that observed with the cytochrome b reductase fragment of nitrate reductase (8).2 In the course of this equilibrium titration, there is no evidence for accumulation of either anionic or neutral flavin semiquinone. Since NADH is an obligatory two-electron donor, this observation indicates not only that the semiquinone is thermodynamically destabilized (i.e. that the quinone/semiquinone couple is lower than the semiquinone/hydroquinone couple) but that dismutation of the two-electron reduced enzyme initially generated by reaction of NADH with the flavin center (possessing reduced heme and flavin semiquinone) is rapid.

The potential of the cytochrome b+/cytochrome b− couple has been determined by the method described by Massey (19) in which a reductive titration of an unknown (a redox center in a protein) is carried out in the presence of an equivalent amount of a standard (a redox dye for which the reduction potential is known). A plot of the logarithm of oxidized/reduced concentrations of the unknown versus the logarithm of the oxidized/reduced concentration of the standard dye (22), in this case, 5-hydroxy-1,4-naphthoquinone (∆E′ = −3 mV) (Fig. 3) yields a reduction potential for the cytochrome b of 15 mV. This value is comparable to that reported by Cannons et al. (12) for the recombinant cytochrome b domain of nitrate reductase from C. vulgaris but is considerably higher than that reported for cytochrome b in holo-nitrate reductases from different sources (23, 24). In the enzyme from C. vulgaris this difference has been attributed to a modulation of the heme reduction potential by the presence of a portion of the molybdopterin-binding region polypeptide (12).

Reaction of Cytochrome c Reductase with Excess NADH—The reaction of oxidized cytochrome c reductase fragment with a pseudo-first-order excess of NADH (that is, under conditions where [NADH] $\gg$ [Cytochrome]) has been examined by rapidly mixing the enzyme with NADH under anaerobic conditions at 10 °C (it being found that lower temperatures are required to slow the reaction sufficiently to be observed in the stopped-flow instrument). Reduction of flavin in these experiments can be monitored conveniently at 460 nm, reduction of the cytochrome b monitored at 556 or 424 nm, and formation of the charge-transfer complex (FADH$_2$-NAD$^+$) monitored at 750 nm. Three
kinetic phases are observed in the course of this reaction (Scheme 1). The fast phase, due to the reduction of the flavin, is complete in 10 ms and is observed as a decrease in absorbance at 460 nm (Fig. 4A). Concomitant with the decrease in absorbance at 460 nm, an increase in absorbance is observed at 750 nm, indicating formation of the charge-transfer complex between the reduced flavin and NADH at the same rate as reduction of flavin. The fast phase of the reaction is found to exhibit hyperbolic dependence on the concentration of NADH, and from a double reciprocal plot a $K_d$ of 3 mM and a $k_{\text{red}}$ of 560 s$^{-1}$ are obtained (Fig. 4A, inset). These values are in agreement with previous studies of the reaction of the corn cytochrome b reductase fragment with NADH (Table I) (8), indicating that the initial reduction of the flavin by NADH is largely independent of the presence of the heme domain.

The spectral change associated with the intermediate phase, with a large absorbance change in the Soret and an absorbance increase at 556 nm, indicates that the process involves the transfer of one electron from the flavin to the cytochrome b (Fig. 4B), with a rate constant of 12 s$^{-1}$; this rate constant is independent of NADH concentration. Breakdown of the charge-transfer complex, as reflected in loss of long wavelength absorbance, accompanies heme reduction, suggesting strongly that electron transfer from the flavin to the cytochrome b is limited by the breakdown of the charge-transfer complex formed between reduced flavin and NADH$^+$ (presumably via product dissociation; see below). The slow phase of the reaction is manifested as a subsequent increase in absorbance at both 556 nm (Fig. 4B) and 750 nm (Fig. 5A) and reflects further heme reduction and accumulation of the charge-transfer complex. The most likely mechanism whereby this occurs is via a dismutation of the flavin semiquinone (remaining after transfer of one electron to the heme) to give a mixture of fully oxidized and fully reduced flavin, followed by the reaction of the former with a second equivalent of NADH to regenerate the charge-transfer complex. The dismutation of the flavin semiquinone is not described by a single exponential, but rather is a strictly second-order reaction with a rate constant of $2.11 \times 10^6$ M$^{-1}$ s$^{-1}$, indicating that the dismutation is a

**FIG. 3. Potentiometric titration of the cytochrome b domain.** The concentration of enzyme used ranged between 15 and 20 $\mu$M. The enzyme was incubated under anaerobic conditions with an equal concentration of 5-hydroxy-1,4-naphthoquinone, 1.6 $\mu$M methyl viologen, and with 250 $\mu$M xanthine and 50 nM xanthine oxidase. Enzyme reduction was monitored at 382 nm, an isosbestic point in the reduction of the dye, and the dye was monitored at 350 nm, an isosbestic point in the reduction of the enzyme. The data are plotted as described by Minnaert (22).

**SCHEME 1.**

$\begin{align*}
| & FAD \rightarrow \text{Cyt b}_\text{oxy} \rightarrow \text{FADH}_2 \rightarrow \text{Cyt b}_\text{red} \\
& k_{\text{fast}} \quad 548 \text{sec}^{-1} \quad 12 \text{sec}^{-1} \quad 2 \times 10^7 \text{M}^{-1} \text{sec}^{-1} \\
& \text{NAD} \rightarrow \text{FADH}_2 - \text{NAD}^+ \rightarrow \text{Cyt b}_\text{red} \\
& k_{\text{intermediate}} \quad k_{\text{slow}} \\
& \text{Cyt b}_\text{red} \\
& \text{FAD}^{-} \rightarrow \text{FAD}^{-} \rightarrow \text{FAD}^{-} \\
& k_{\text{red}} \quad k_{\text{slow}}
\end{align*}$
Reaction of Cytochrome c Reductase with One Equivalent of NADH—Reaction of the cytochrome c reductase fragment with one equivalent of NADH (approximately 14 μM each) is again triphasic. The absorbance change associated with the fast phase is essentially identical to that seen in the reaction with excess NADH, with an absorbance decrease at 460 nm and an increase in absorbance at 750 nm, indicating flavin reduction and simultaneous formation of the charge-transfer complex (data not shown). The intermediate kinetic phase representing the one electron reduction of cytochrome b is also observed, again as an increase in absorbance at 556 nm and an absorbance decrease at 750 nm. The absorbance associated with the slow phase of the reaction under conditions of stoichiometric NADH, however, is quite distinct from that observed in the presence of excess NADH (Fig. 5B). Under conditions of excess NADH, dismutation is followed by re-reduction of the 50% of the protein containing oxidized flavin and quantitative accumulation of the charge-transfer complex. With stoichiometric

bimolecular process.3

Reaction of Cytochrome c Reductase with One Equivalent of NADH—Reaction of the cytochrome c reductase fragment with one equivalent of NADH (approximately 14 μM each) is again triphasic. The absorbance change associated with the fast phase is essentially identical to that seen in the reaction with excess NADH, with an absorbance decrease at 460 nm and an increase in absorbance at 750 nm, indicating flavin reduction and simultaneous formation of the charge-transfer complex (data not shown). The intermediate kinetic phase representing the one electron reduction of cytochrome b is also observed, again as an increase in absorbance at 556 nm and an absorbance decrease at 750 nm. The absorbance associated with the slow phase of the reaction under conditions of stoichiometric NADH, however, is quite distinct from that observed in the presence of excess NADH (Fig. 5B). Under conditions of excess NADH, dismutation is followed by re-reduction of the 50% of the protein containing oxidized flavin and quantitative accumulation of the charge-transfer complex. With stoichiometric

CONCLUSIONS

The visible absorption spectrum of the recombinant cytochrome c reductase fragment of spinach nitrate reductase is, as expected, dominated by the cytochrome b cofactor and is virtu-

Fig. 5. Time course of the formation and decay of the charge-transfer complex between reduced flavin and NADH. A, 11 μM enzyme was mixed rapidly with 50 μM NADH and the formation of the charge-transfer complex monitored at 750 nm. B, 14 μM enzyme was mixed rapidly with 14 μM NADH and the formation and decay of the charge-transfer complex monitored at 750 nm. Both reactions were performed under anaerobic conditions at 10 °C.

Fig. 6. EPR spectrum of the flavin semiquinone. 42 μM enzyme was mixed with 42 μM NADH in a rapid-quench apparatus under anaerobic conditions at 2 °C. EPR samples were collected as described under “Materials and Methods.” The EPR spectrum was obtained at 150 K using 10 mW power and 5 G modulation.

NADH, this re-reduction cannot take place and the dismutation reaction leaves a 1:1 mixture of enzyme possessing fully oxidized and fully reduced flavin (each with the heme center reduced). The former species cannot form the charge-complex because the flavin is in the incorrect oxidation state. The latter species can (and does) form a certain amount of charge-transfer complex, as reflected in the transient shown in Fig. 5B, which does not return to zero absorbance at the end of the slow phase. The incomplete formation of charge-transfer complex with the fully reduced enzyme is presumably due to the relatively low concentration of NADH at the conclusion of the reaction. It is to be noted that to the extent the dismutation reaction is incomplete, the two-electron reduced enzyme that remains will also be unable to form the charge-transfer complex, as the electron distribution within the protein favors reduced heme and flavin semiquinone over oxidized heme and flavin hydroquinone (as reflected in the large spectral change associated with the intermediate phase of the reaction with NADH whose wavelength dependence indicates that the process reflects heme reduction at the expense of the initially formed flavin hydroquinone). Again the flavin is in the incorrect oxidation state for formation of the charge-transfer complex. The principal factor which determines whether the charge-transfer complex is formed in the experiments with excess or stoichiometric [NADH] is its kinetic (as opposed to thermodynamic) stability.

In order to examine the presumed flavin semiquinone species generated upon electron transfer from the flavin hydroquinone to the heme (i.e. that formed at completion of the second phase of the reaction), a quench experiment was performed, aimed at stopping the reaction of the cytochrome c reductase fragment with NADH prior to dismutation of the flavin semiquinone. The cytochrome c reductase fragment was rapidly mixed with one equivalent of NADH in a rapid quench apparatus, and at 4 °C and samples were collected directly into EPR tubes and frozen immediately in a dry ice/acetone bath. The EPR spectrum of the flavin semiquinone (Fig. 6) yielded a signal with a line width of approximately 13 G, indicating the accumulation of the anionic form of flavin semiquinone.

FIG. 6. EPR spectrum of the flavin semiquinone. 42 μM enzyme was mixed with 42 μM NADH in a rapid-quench apparatus under anaerobic conditions at 2 °C. EPR samples were collected as described under “Materials and Methods.” The EPR spectrum was obtained at 150 K using 10 mW power and 5 G modulation.

NADH, this re-reduction cannot take place and the dismutation reaction leaves a 1:1 mixture of enzyme possessing fully oxidized and fully reduced flavin (each with the heme center reduced). The former species cannot form the charge-complex because the flavin is in the incorrect oxidation state. The latter species can (and does) form a certain amount of charge-transfer complex, as reflected in the transient shown in Fig. 5B, which does not return to zero absorbance at the end of the slow phase. The incomplete formation of charge-transfer complex with the fully reduced enzyme is presumably due to the relatively low concentration of NADH at the conclusion of the reaction. It is to be noted that to the extent the dismutation reaction is incomplete, the two-electron reduced enzyme that remains will also be unable to form the charge-transfer complex, as the electron distribution within the protein favors reduced heme and flavin semiquinone over oxidized heme and flavin hydroquinone (as reflected in the large spectral change associated with the intermediate phase of the reaction with NADH whose wavelength dependence indicates that the process reflects heme reduction at the expense of the initially formed flavin hydroquinone). Again the flavin is in the incorrect oxidation state for formation of the charge-transfer complex. The principal factor which determines whether the charge-transfer complex is formed in the experiments with excess or stoichiometric [NADH] is its kinetic (as opposed to thermodynamic) stability.

In order to examine the presumed flavin semiquinone species generated upon electron transfer from the flavin hydroquinone to the heme (i.e. that formed at completion of the second phase of the reaction), a quench experiment was performed, aimed at stopping the reaction of the cytochrome c reductase fragment with NADH prior to dismutation of the flavin semiquinone. The cytochrome c reductase fragment was rapidly mixed with one equivalent of NADH in a rapid quench apparatus, and at 4 °C and samples were collected directly into EPR tubes and frozen immediately in a dry ice/acetone bath. The EPR spectrum of the flavin semiquinone (Fig. 6) yielded a signal with a line width of approximately 13 G, indicating the accumulation of the anionic form of flavin semiquinone.

CONCLUSIONS

The visible absorption spectrum of the recombinant cytochrome c reductase fragment of spinach nitrate reductase is, as expected, dominated by the cytochrome b cofactor and is virtu-

3 From the standpoint of chemical kinetics, the dismutation reaction for CcR2e is of the form 2A → B + C. This is a special case of second-order reaction in which the integrated rate equation is of the form \( t/[A] = kt + 1/[A] \), and in the present case the rate constant is obtained from the slope of a plot of \( \Delta \text{Absorbance}/\Delta \text{Time vs. time} \) (25). The slope of this plot is independent of the initial concentration of species A.
ally indistinguishable from that reported for the native enzyme (2). This indicates that the contribution from the molybdenum domain to the absorption spectrum of native enzyme is extremely small. This observation is consistent with the results reported for the enzyme sulfite oxidase (26), whose molybdenum domain exhibits high degree of sequence homology with that of nitrate reductase. The molybdenum domain of sulfite oxidase exhibits two broad bands at around 350 nm and between 450 and 500 nm, with an extinction coefficient of about 1600 mM⁻¹ cm⁻¹ at 480 nm. The CD spectra of oxidized and reduced Cytochrome b reductase reported here are also consistent with those reported for the cytochrome b reductase fragment of corn nitrate reductase (8) and the flavin and heme domains of nitrate reductase from *C. vulgaris* (12). The CD spectrum of the oxidized enzyme is predominantly due to the flavin domain, whereas that of the reduced enzyme is principally due to the cytochrome b. Particularly in the case of the flavin domain, the close similarity of the flavin contribution to the CD of oxidized the flavin-containing cytochrome b reductase fragment of nitrate reductase (8), CcR (present work), and native nitrate reductase (12) suggests that there is little perturbation of the flavin environment in going from one protein form to another.

The reduction potential determined for the cytochrome b of the cytochrome c reductase fragment (ΔE = 15 mV) (Table I) is considerably higher than that reported for native nitrate reductase (ΔE = -123 mV) (23) but corresponds well to the value reported by Cannons et al. for the heme domain of the nitrate reductase from *C. vulgaris* (ΔE = +16 mV) (12). Cannons et al. (12) have demonstrated that while the cytochrome b domain in isolation has a rather high potential, increasing the size of the cytochrome b domain to include a part of the molybdenum binding portion of the protein results in a decrease in the potential of the cytochrome b by 44 mV, suggesting that residues in the molybdenum domain are involved in modulating the cytochrome b reduction potential. Our results indicate that there is no comparable effect seen with the flavin domain and that the cytochrome b reduction potential in the cytochrome c reductase fragment is similar to that of the isolated cytochrome b. The heme potential being so much higher than the flavin potential (ΔE = -280 mV) (23) is consistent with the observation that during equilibrium reductive titrations with either sodium dithionite or NADH the heme is reduced prior to the flavin (present work).

The reaction of the cytochrome c reductase fragment of nitrate reductase with NADH is found to be triphasic (Scheme 1), with the three phases representing flavin reduction (fast phase), electron transfer from reduced flavin to the heme concomitant with breakdown of the FADH₂-NAD⁺ charge-transfer complex (intermediate phase), and dismutation of the two-electron reduced enzyme to yield a mix of one- and three-electron reduced enzyme (slow phase, followed by reaction of the one-electron reduced enzyme with NADH (24, 25)). These phases are shown in Table I and indicate that the presence of the heme domain has little effect on the reaction of NADH with the flavin. After flavin reduction, electron transfer to cytochrome b takes place at a relatively slow rate (12 s⁻¹) and is apparently rate-limited by the breakdown of the charge-transfer complex. Since electron transfer from the flavin to the heme is highly favorable in the cytochrome c reductase fragment even in the presence of NAD⁺ (the difference in potentials is ~300 mV), this effect is undoubtedly kinetic rather than thermodynamic in nature. Even so, under conditions of even a moderate excess of NADH, the relative rate constants associated with formation and decay of the charge-transfer complex are such that essentially 100% of the protein transiently accumulates as the long wavelength-absorbing species.

Subsequent to heme reduction the flavin semiquinone that is left dismutates to give a 1:1 mixture of cytochrome c reductase fragment possessing reduced heme plus either fully oxidized or fully reduced flavin. In the presence of excess NADH, the former species is very rapidly reduced by a second equivalent of NADH resulting in 100% formation of the FADH₂-NAD⁺ charge-transfer complex (observed as an increase in absorbance at 750 nm at longer reaction times; Fig. 5A). In the reaction of the cytochrome c reductase fragment with a stoichiometric amount of NADH, this reaction is not observed (Fig. 5B), and the slow phase is instead represented simply by the relatively small change in absorbance due to the dismutation of the flavin semiquinone. The thermodynamic favorability of this dismutation indicates that the reduction potential for the FAD/FAD⁺ couple is substantially lower than that of FAD/FAD⁺ couple and is consistent with the fact that during a reductive titration of the cytochrome b reductase fragment of nitrate reductase (which contains only the flavin cofactor) with sodium dithionite, formation of the flavin semiquinone does not accumulate to an appreciable extent (8). Dismutation of the flavin semiquinone is also seen in related flavoenzymes such as cytochrome b₅₆₅ reductase (30, 31) and adrenodoxin reductase (32). In the case of cytochrome b₅₆₅ reductase, dismutation occurs only in the absence of the product NAD⁺, with the semiquinone species being stabilized in the presence of product (but not as a charge-transfer complex). The behavior of the cytochrome c reductase fragment of spinach nitrate reductase is similar to that of adrenodoxin reductase in that it does not stabilize the flavin semiquinone even in the presence of NAD⁺, and electron transfer from the reduced flavin to cytochrome b does not take place prior to breakdown of the enzyme-product complex. On the basis of a 13-G line width in the EPR signal of the flavin semiquinone (Fig. 6) observed in the two-electron reduced cytochrome c reductase fragment is of the red anionic type, as has been observed for native nitrate reductase (21).

Given the difference in potentials between the two centers in the cytochrome c reductase fragment of spinach nitrate reductase, reduction of the cytochrome b by transfer of one electron from FADH₂ is highly favorable. Also, given the expected proximity of the two centers (14), the intrinsic rate of electron transfer is expected to be very fast compared to turnover and not in any way rate-limiting. However, in the reaction of the cytochrome c reductase fragment with NADH, electron transfer is found to be relatively slow (12 s⁻¹), and the behavior of the system is consistent with electron transfer being limited by the breakdown of the charge-transfer complex between reduced flavin and NAD⁺. Since dissociation of the product from the FADH₂-NAD⁺ complex is an obligatory step in the course of turnover, this process likely represents the rate-limiting step in overall catalysis of nitrate reductase, although studies of the holo-nitrate reductase are required to definitively establish this. Indeed, the 12 s⁻¹ seen here for the intermediate phase of the reaction (at 10 °C) compares fairly well with the *k*ₕₐₜ of ~60 s⁻¹ observed with native spinach nitrate reductase under comparable conditions but at 25 °C (33).

Previous studies with the cytochrome b reductase fragment...
of nitrate reductase have implicated the invariant cysteine 242 residue as playing an important but not essential role in the reductive half-reaction of the catalytic cycle (7). A comparison of the kinetic properties of the wild-type fragment with a C242S mutant indicated that the rate of reduction of the flavin decreased 7-fold on mutating the cysteine to a serine, and this was interpreted to mean that the cysteine played a role principally in facilitating electron transfer from NADH to the flavin (8). In light of the current results with the cytochrome c reductase fragment indicating that NAD$^+$ dissociation is rate-limiting, it is possible that an equally important catalytic role of cysteine 242 is to facilitate NADH release from the charge-transfer complex formed at the completion of the fast phase of the reaction. It remains for future experiments to establish whether this is the reason that cysteine 242 is invariant among plant nitrate reductases.

Acknowledgments—We thank Dr. T. Suzuki and Prof. Toshi Yubisui, Department of Biology, Kochi University, Kochi, Japan for carrying out protein sequencing on the recombinant CcR and Craig F. Hemann for help with the circular dichroism studies.

REFERENCES

1. Campbell, W. H. (1989) in "Molecular and Genetic Aspects of Nitrate Assimilation" (Wray, J. L., and Kinghorn, J. R., eds) pp. 125–154, Oxford University Press, New York
2. Solomonsen, L. P., and Barber, M. J. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 225–253
3. Rouze, P., and Caboche, M. (1992) in "Society for Experimental Biology Seminar Series 49" (Wray, J. L., ed) pp. 45–77, Cambridge University Press, Cambridge
4. Campbell, W. H., and Kinghorn, J. R. (1990) Trends Biochem. Sci. 15, 315–319
5. Crawford, N. M., Smith, M., Bellissimo, D., and Davis, R. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8673–8676
6. Barber, M. J., and Neame, P. J. (1990) J. Biol. Chem. 265, 20912–20915
7. Dwivedi, U. N., Shiraiishi, N., and Campbell, W. H. (1994) J. Biol. Chem. 269, 13785–13791
8. Ratnam, K., Shiraiishi, N., Campbell, W. H., and Hille, R. (1995) J. Biol. Chem. 270, 24067–24072
9. Lu, G., Campbell, W. H., Schneider, G., and Lindqvist, Y. (1994) Structure 2, 809–821
10. Kubo, Y., Ogura, N., and Nakagawa, H. (1988) J. Biol. Chem. 263, 19684–19689
11. Hyde, G. E., and Campbell, W. H. (1990) Biochem. Biophys. Res. Commun. 188, 1285–1291
12. Cannone, A. C., Barber, M. J., and Solomonsen, L. P. (1993) J. Biol. Chem. 268, 3268–3271
13. Campbell, W. H. (1992) Plant Physiol. 99, 693–699
14. Lu, G., Lindqvist, Y., Schneider, G., Dwivedi, U., and Campbell, W. H. (1995) J. Mol. Biol. 248, 931–948
15. Xia, Z.-X., and Matthews, F. S. (1990) J. Mol. Biol. 212, 837–863
16. Hyde, G. E., Crawford, N. M., and Campbell, W. H. (1991) J. Biol. Chem. 266, 23542–23547
17. Cregg, J. M., Barringer, K. J., and Hessler, A. Y. (1985) Mol. Cell. Biol. 5, 3376–3385
18. Burleigh, B. D., Foust, G. P., and Williams, C. H. (1969) Anal. Biochem. 84, 75–81
19. Massey, V. (1990) in "Flavins and Flavoproteins" (Curti, B., Ronchi, S., and Zanetti, G., eds) pp. 59–67, Walter de Gruyter, Berlin
20. Clark, W. M. (1972) Oxidation Reduction Potentials of Organic Systems, p. 415, R. E. Krieger, Huntington, NY
21. Kay, C. J., Barber, M. J., and Solomonsen, L. P. (1988) Biochemistry 27, 6142–6149
22. Minnaert, K. (1965) Biochim. Biophys. Acta 110, 42–56
23. Kay, C. J., Barber, M. J., Notton, B. A., and Solomonsen, L. P. (1989) Biochem. J. 263, 285–287
24. Kay, C. J., Solomonsen, L. P., and Barber, M. J. (1986) J. Biol. Chem. 261, 5799–5802
25. Moore, J. W., and Pearson, R. G. (1981) Kinetics and Mechanism, 3rd Ed., p. 19, John Wiley and Sons, New York
26. Johnson, J. L., and Rajagopalan, K. V. (1977) J. Biol. Chem. 252, 2017–2025
27. Solomonsen, L. P., and Barber, M. J. (1984) in "Flavins and Flavoproteins" (Bray, R. C., Engel, P. C., and Mayhew, S. G., eds) pp. 47–50, Walter de Gruyter, Berlin
28. Aliverti, A., Piubelli, L., Zanetti, G., Luberstedt, T., Herrmann, R. G., and Curti, B. (1995) Biochemistry 34, 6374–6380
29. Williams, C. H. (1976) in "The Enzymes" (Boyer, P. D., ed.), Vol. 8, pp. 154–165, Academic Press, New York
30. Iyanagi, T., Watanabe, S., and Anan, K. (1984) Biochemistry 23, 8374–8380
31. Meyer, T. E., Shirabe, K., Yubisui, T., Takeshita, M., Bes, M. T., Cusanovich, M. A., and Tollin, G. (1995) Arch. Biochem. Biophys. 318, 457–464
32. Kobayashi, K., Miura, S., Miki, M., Ichikawa, Y., and Tagawa, S. (1995) Biochemistry 34, 12932–12936
33. Barber, M. J., Notton, B. A., Kay, C. J., and Solomonsen, L. P. (1989) Plant Physiol. 90, 70–74