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Kinetics and Reactivity of the Flavin and Heme Cofactors of Cellobiose Dehydrogenase from *Phanerochaete chrysosporium*

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ABSTRACT: The flavin cofactor within cellobiose dehydrogenase (CDH) was found to be responsible for the reduction of all electron acceptors tested. This includes cytochrome *c*, the reduction of which has been reported to be by the reduced heme of CDH. The heme group was shown to affect the reactivity and activation energy with respect to individual electron acceptors, but the heme group was not involved in the direct transfer of electrons to substrate. A complicated interaction was found to exist between the flavin and heme of cellobiose dehydrogenase. The addition of electron acceptors was shown to increase the rate of flavin reduction and the electron transfer rate between the flavin and heme. All electron acceptors tested appeared to be reduced by the flavin domain. The addition of ferric iron eliminated the flavin radical present in reduced CDH, as detected by low temperature ESR spectroscopy, while it increased the flavin radical ESR signal in the independent flavin domain, more commonly referred to as cellobiose: quinone oxidoreductase (CBQR). Conversely, no radical was detected with either CDH or CBQR upon the addition of methyl-1,4-benzoquinone. Similar reaction rates and activation energies were determined for methyl-1,4-benzoquinone with both CDH and CBQR, whereas the rate of iron reduction by CDH was five times higher than by CBQR, and its activation energy was 38 kJ/mol lower than that of CBQR. Oxygen, which may be reduced by either one or two electrons, was found to behave like a two-electron acceptor. Superoxide production was found only upon the inclusion of iron. Additionally, information is presented indicating that the site of substrate reduction may be in the cleft between the flavin and heme domains.

A cellobiose dehydrogenase (CDH)[1] is secreted by *Phanerochaete chrysosporium* when grown using cellulose as the nutrient carbon source (*1*, *2*). Originally, it was believed that there were two cellobiose-dependent enzymes, a hemoflavoprotein named cellobiose oxidase and a flavoprotein named cellobiose:quinone oxidoreductase (CBQR). However, in 1992, Kremer and Wood (*3*) conclusively showed that only one protein existed, which upon proteolytic cleavage gave an inactive heme fragment and a flavin fragment, CBQR, which retains many of the properties of intact CDH.

Cellobiose dehydrogenase is a two-domain hemoflavoenzyme that uses cellobiose as an electron donor for the reduction of a wide range of electron acceptors. While the physiological electron acceptor is unknown, those most likely to have physiological relevance are quinones, molecular oxygen, Fe^{3+}, Mn^{3+}, and phenoxyl radicals. There has been much debate in the literature concerning the physiological function of CDH and no less than nine hypotheses are in the literature (*4*−*15*). Our laboratory has recently published several papers concerning the free radicals produced by CDH and its involvement in the biodegradation of chemicals by *P. chrysosporium* (*16*−*19*).

Cellobiose dehydrogenase contains a flavin (FAD) and a β-type heme group which are located in separate domains connected by a short flexible linker (*20*). It is known that cellobiose is oxidized by two electrons at a site located in the flavin domain. The reduced flavin subsequently passes one electron to the heme, resulting in the formation of a flavin radical and reduced heme (*21*). Some electron acceptors such as quinones and molecular oxygen are reduced at similar rates by intact CDH and by the proteolytic fragment, CBQR. Others, such as cytochrome *c*, are only reduced at significant rates by intact enzyme. In general, the rate of reduction of one-electron acceptors, such as iron and copper, decrease upon cleavage of the two domains while the rates of reduction of most two-electron acceptors, such as quinones, are minimally affected.

It has been previously reported and is generally accepted that most electron acceptors are reduced at the flavin domain with the exception being cytochrome *c*. Reduction of cytochrome *c* was reported to require intact CDH with the electron being passed from the reduced heme group of CDH to the heme of cytochrome *c* (*22*, *23*). We offer evidence that suggests cytochrome *c* is reduced by the flavin radical of CDH and an explanation of earlier results.

Aside from the initial reduction of CDH by cellobiose, little is known about the electron transfer between the domains, and nothing has been published regarding the influence of individual electron acceptors. We examined the electron transfer between the domains of CDH utilizing ESR

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[1] Abbreviations: CDH, cellobiose dehydrogenase; CBQR, cellobiose: quinone oxidoreductase; MBQ, methyl-1,4-benzoquinone; SOD, superoxide dismutase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

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and stopped-flow spectroscopy techniques using ferric iron and methyl-1,4-benzoquinone (MBQ) as model one- and two-electron acceptors. We have also determined the activation energies for ferric iron and MBQ reduction by CDH and CBQR. Studies with CBQR were done using enzyme obtained by the proteolysis of CDH by both the physiological protease and papain, as described by Henriksson et al. (*24*).

MATERIALS AND METHODS

Chemicals Used. All chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) and were of the highest purity available. All stock solutions were prepared using purified water with a specific resistance greater than 17.5 MΩ, and all buffers were chromatographed through a Chelex 100 column (1 × 10 cm).

Production and Purification of CDH. Cultures of *P. chrysosporium* BKMF-1767 were grown, and CDH was purified as previously described (*16*). Briefly, the fungus was grown in 1 L shaking cultures for 6 days at 37 °C. The extracellular fluid was concentrated 60-fold by ultrafiltration. CDH was precipitated using 365 g/L ammonium sulfate and resuspended in 10 mM acetate buffer, pH 5.0. The solution was chromatographed using a DEAE-Sepharose fast flow column (2.5 cm × 30 cm) and a linear gradient of 0 to 1 M NaCl in 10 mM acetate buffer, pH 5.0. Fractions exhibiting CDH activity were pooled and chromatographed using a phenyl-Toyopearl column (2.5 cm × 30 cm, Bio-Rad Laboratories, Hercules, CA) and a reverse gradient of 2.5 to 0 M NaCl. Fractions exhibiting CDH activity were concentrated and chromatographed using a Bio-Gel P-150 column (2.5 cm × 80 cm) and 10 mM acetate buffer, pH 5.0. The ratio of protein absorbance at 280 to heme absorbance at 420 nm for the purified enzyme was typically 1.65−1.69. Purified CDH was verified by SDS−PAGE and migrated as a single band with an approximate molecular weight of 90 000.

Purification of CBQR. The independent flavin domain, CBQR, was purified from cultures of *P. chrysosporium* using the same purification method described above. CBQR and CDH were separated during the DEAE-Sepharose Fast Flow column step described above. CBQR was also produced by papain cleavage of CDH using the method described by Henriksson et al. (*26*). The ratio of protein absorbance at 280 to flavin absorbance at 458 nm for the purified enzyme was typically 12.5 and one protein band was observed by SDS−PAGE with an approximate molecular weight of 55 000.

Enzyme Assay. Cellobiose dehydrogenase activity was monitored at 25 °C using 0.1 mM 2,6-dichloroindophenol and 1 mM cellobiose in 50 mM acetate buffer, pH 4.5. Activity was monitored as the production of 2,6-dichlorophenol indophenol at 522 nm and rates were calculated using 6900 M^{-1} cm^{-1} as the molar extinction coefficient. Alternatively, activity was monitored using 100 μM cytochrome *c* at 550 nm or with 1 mM ferric chloride and 1 mM ferrozine monitored at 564 nm. Rates were calculated using the extinction coefficients of 19 600 M^{-1} cm^{-1} for cytochrome *c* reduction and 27 900 for the ferrozine−ferrous iron complex.

ESR Spectroscopy. Low-temperature ESR spectra were obtained using a Bruker ESP-300E spectrometer with the following parameters: temperature, 8 K; microwave power, 20 mW; microwave frequency, 9.64 GHz; modulation amplitude, 12.63 G; modulation frequency, 100 kHz; sweep time, 20.9 s; scans, 5.

The superoxide radical was trapped using DMPO. Reaction conditions were 1 mM cellobiose and 50 mM DMPO in 50 mM acetate buffer, pH 4.5. Reactions were initiated with 10 μM CDH. Ferric iron was added in various concentrations ranging from 1 to 100 μM. The effects of 1 mM Desferal, 1000 units of catalase, and 1000 units of superoxide dismutase were also examined. All spin trap-radical adduct ESR spectra were recorded at room temperature using a Bruker ECS 106 ESR spectrometer. The following parameters were used: temperature, 25 °C; microwave power, 20 mW; microwave frequency, 9.62 GHz; modulation amplitude, 1.06 G; modulation frequency, 50 kHz; sweep time, 80 s; scans, 3.

Stopped-Flow Spectroscopy. All experiments using stopped flow spectroscopy were done in 50 mM acetate buffer, pH 4.5, using a KinTek SF-2001 spectrometer at 25 °C. The concentrations of CDH and CBQR after mixing were 5 μM. The reduction of the heme prosthetic group was monitored as an increased absorbance at 562 nm. The reduction of the flavin was monitored as a decreased absorbance at 458 nm. All concentrations are given in the figure legends. Rates were calculated using the difference in the extinction coefficients of oxidized and reduced CDH at 562 nm (15 000 M^{-1} cm^{-1}) and 458 nm (8900 M^{-1} cm^{-1}).

Determination of Activation Energies. Activation energies were determined for the reduction of ferric iron and MBQ by determining activities at different temperatures using 0.3 μM CDH or CBQR, 1 mM cellobiose, and either 1 mM MBQ or 1 mM ferric iron and 1 mM ferrozine in 50 mM acetate buffer, pH 4.5,. The data were plotted as log(*k*) vs 1/*T* in kelvin. Activation energies were calculated using the equation slope $= -E_A/2.3R$ where *R* is the gas constant.

RESULTS

The visible spectrum of CDH, Figure 1A, is dominated by the heme prosthetic group, which has absorbance maxima at 420, 529, and 570 nm in the oxidized form, that shift to 428, 533, and 562 nm upon reduction by cellobiose. The absorbance maxima for the flavin in oxidized CBQR, Figure 1B, are at 385 and 458 nm. Upon reduction of CBQR, one peak was detected with a maxima at 355 nm. Upon reduction of CDH, the increase in absorbance at 562 nm and the decrease at 458 nm can be attributed to reduction of the heme and flavin, respectively. These wavelengths were used in later stopped-flow experiments to determine the oxidation state of the cofactors. Ferric iron and MBQ were chosen as model one- and two-electron acceptors because they were reduced by both CDH and CBQR. Additionally, both had minimal effects on absorption at 458 and 562 nm in their oxidized or reduced forms, thus allowing for monitoring of flavin and heme reduction at these wavelengths.

Comparison of CBQR isolated directly from cultures or produced proteolytically by papain cleavage showed minimal differences with respect to the reduction rates of ferric iron and MBQ, and neither had appreciable cytochrome *c* reductase activity. There was, however, a difference in the interaction between the independent flavin and heme do-

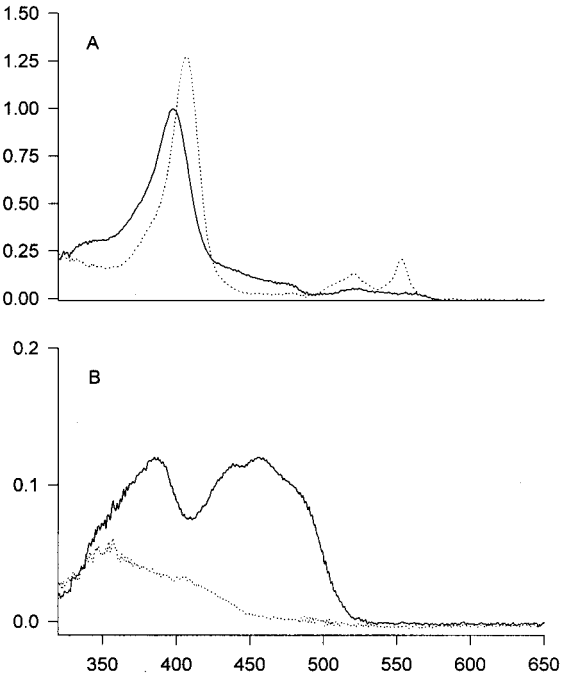

FIGURE 1: UV-vis absorption spectra of purified CDH (A) and CBQR (B). Oxidized (solid line) and reduced (dashed line) spectra of CDH and CBQR at pH 4.5 and 10 μM enzyme.

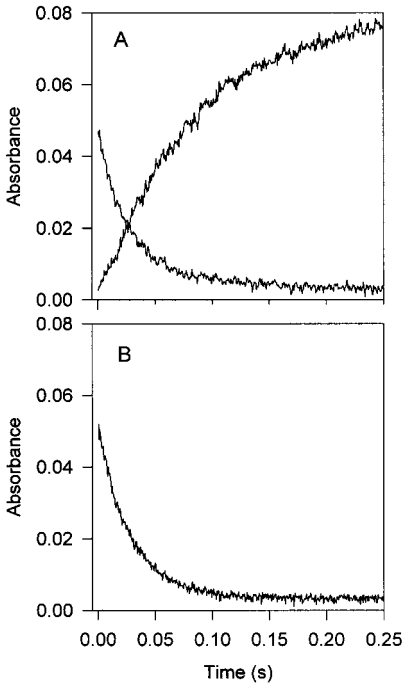

FIGURE 2: Time-course of the reduction of CDH (A) and CBQR (B) by cellobiose. Reduction of the flavin cofactor was monitored by the decrease in absorbance at 458 nm and heme reduction was monitored by the increase in absorbance at 562 nm upon the addition of 1 mM cellobiose and 5 μM enzyme in 50 mM acetate buffer, pH 4.5.

mains. The independent flavin and heme domains isolated from fungal cultures were not able to interact in a manner that the reduced flavin could facilitate reduction of the heme. However, flavin and heme domains produced through papain proteolysis readily interacted and the heme was reduced upon the addition of cellobiose (data not shown). This difference did not effect the results in this study, but may be important under certain conditions.

Reduction of CDH and CBQR by saturating amounts of cellobiose were monitored by stopped-flow spectroscopy. Both the flavin and heme prosthetic groups of CDH were reduced by cellobiose at pH 4.5, Figure 2. Increasing the pH to 6.0 had little effect on the rate of flavin reduction by cellobiose in CDH or CBQR. However, higher pH significantly slowed the rate of electron transfer from the flavin to the heme in CDH (data not shown). Altering the pH allowed the observation of both the fully reduced flavin and the flavin semiquinone. This correlates well with the earlier work of Samejima et al. (*25*).

The addition of iron caused an increase in the rate of flavin reduction and electron transfer between the flavin and heme of CDH, as determined by stopped-flow spectroscopy, Table 1. Addition of MBQ caused an increase in the rate of flavin reduction, but the rate of electron transfer to the heme was unaffected. The kinetic data were found to be biphasic, but only the initial fast phase was considered. There was no obvious connection between reaction conditions and calculated values for the second, slow, phase. To the best of our knowledge, this is the first report showing that the presence of electron acceptors alters the reactivity of the cofactors of CDH.

Cytochrome *c* reduction was inhibited by high concentrations of cellobiose. The rate of cytochrome *c* reduction by CDH was found to be 16 s^{-1} using either 100 μM or 1 mM cellobiose. When the concentration of cellobiose was increased to 10 mM, the rate of cytochrome *c* reduction was

Table 1: Rate Constants of FAD and Heme Reduction of CDH in the Presence of Ferric Iron or MBQ

CDH +	rate of FAD reduction (s^{-1})[b]	rate of heme reduction (s^{-1})[b]
-	74 ± 3	16 ± 1
10 μM MBQ	80 ± 4	16 ± 1
50 μM MBQ	134 ± 3	17 ± 1
100 μM MBQ	206 ± 9	15 ± 1
1000 μM MBQ	*a*	23 ± 2
100 μM Fe^{3+}	88 ± 6	35 ± 2
250 μM Fe^{3+}	152 ± 3	86 ± 3
500 μM Fe^{3+}	*a*	212 ± 4
1000 μM Fe^{3+}	*a*	206 ± 3

[a] Rate too high for accurate reading. [b] Reduction of the heme iron was monitored by absorption changes at 562 nm whereas FAD reduction was monitored by changes at 458 nm upon the addition of cellobiose, 1 mM, CDH, 5 μM, and various concentrations of ferric chloride or MBQ in 50 mM acetate buffer, pH 4.5.

8 s^{-1}. When 10 mM cellobiose was incubated with CDH under anaerobic conditions for 30 min to fully reduce the enzyme prior to the addition of cytochrome *c*, the rate was found to be 0.7 s^{-1}, Figure 3. CDH incubated anaerobically with cellobiose was found to be fully reduced using cold temperature ESR and was not inactivated during the incubation with cellobiose, as it retained 100% of its initial quinone reductase activity. Inhibition of the cytochrome *c* reductase activity of CDH by high cellobiose concentrations indicates that the FADH radical is responsible for cytochrome *c* reduction, not the reduced heme as reported in previous publications (*22*, *23*).

The rate of ferric iron reduction is five times higher for intact CDH while the rate of MBQ reduction is the same for CDH and CBQR. The activation energies for the reduction of ferric iron and MBQ were compared for CDH

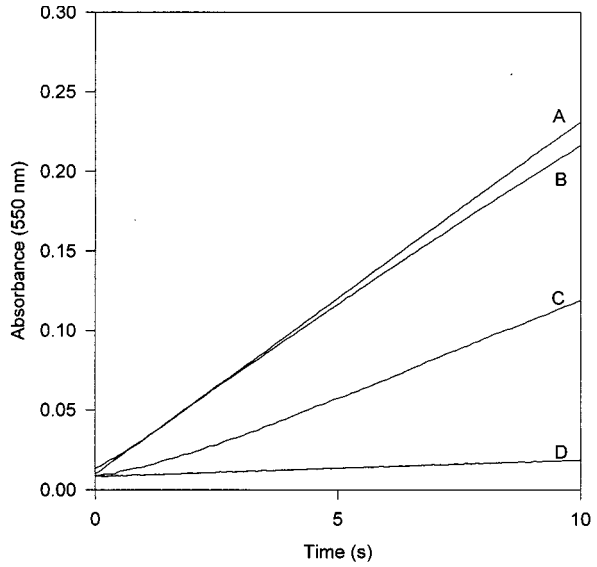

FIGURE 3: Effect of cellobiose on cytochrome *c* reduction. Cytochrome *c* reduction was monitored at 550 nm in incubations that included (A) 0.1 μM CDH, 0.1 mM cellobiose, and 100 μM cytochrome *c* in 50 mM acetate buffer, pH 4.5. Traces B and C were obtained under similar conditions as A, but the concentration of cellobiose were increased to 1 and 10 mM, respectively. Trace D was obtained from an identical incubation as in C, but the CDH was anaerobically preincubated with cellobiose for 30 min. The reaction was initiated by the addition of cytochrome *c*.

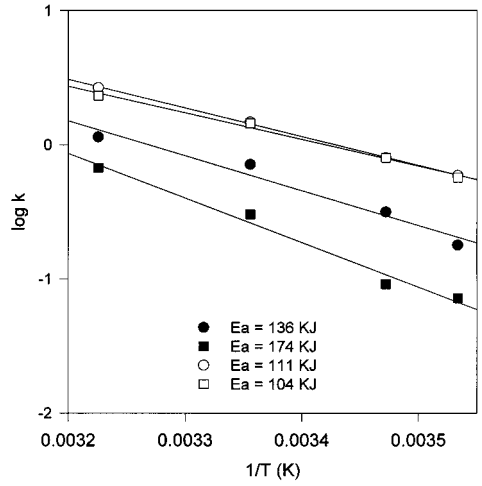

FIGURE 4: Arrhenius plot for reduction of ferric iron and MBQ. The activities of 0.3 μM CDH, closed symbols, or CBQR, open symbols, for the reduction of ferric iron, circles, or MBQ, squares, were determined at 10, 15, 25, and 37 °C. The observed rates were plotted as log(*k*) vs 1/*T*. The activation energies were calculated using the equation slope = $E_A/2.3R$.

and CBQR to see if the heme domain has a greater effect on the activation energy of the one-electron acceptor ferric iron than the two-electron acceptor MBQ. The activation energy for MBQ reduction was found to be 111 and 104 kJ/mol for CDH and CBQR, respectively, while the activation energy for ferric iron reduction was 136 and 174 kJ/mol for CDH and CBQR, respectively (Figure 4).

Cold temperature ESR was used to examine the electronic environment of the heme and flavin radical, with and without substrate present. The cold temperature ESR spectra of the oxidized form of the independent heme domain and CDH, Figure 5, were nearly identical with *G* values of 3.46 and 2.04 for the heme iron. A G_3 signal could not be detected.

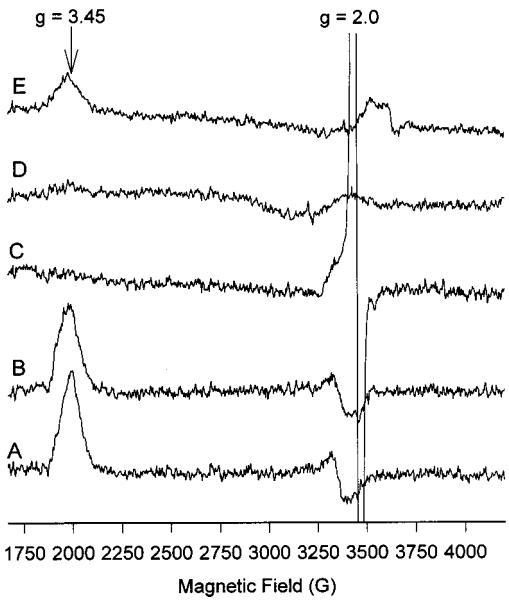

FIGURE 5: Cold temperature ESR spectra (8 K) of CDH and its independent heme domain. Spectrum A was detected using 100 μM of the independent heme domain. Spectrum B was detected using 100 μM CDH. Spectrum C was detected using 100 μM CDH and 10 mM cellobiose. Spectra D and E were detected using similar conditions to spectrum C with the addition of 10 mM ferric chloride or 10 mM methyl-1,4-benzoquinone, respectively.

This is similar to the results published by Morpeth (*26*). Upon the addition of cellobiose, no signal was observed for the heme iron, but a strong flavin radical signal at *G* 2.00 was detected. The addition of iron resulted in the loss of the flavin radical signal and no signal for the heme iron was detected, indicating the heme iron was still reduced. The addition of MBQ resulted in loss of the flavin radical signal and a partial reappearance of the heme iron signal. Additionally, the *G* equals 2.04 value of the heme iron was shifted to *G* equals 1.97. This is most likely due to an alteration occurring in the heme iron environment upon the addition of MBQ.

There was no detectable cold temperature ESR signal for CBQR (Figure 6). Upon reduction by cellobiose, a small signal for the flavin radical was detected at *G* 2.00. The addition of iron resulted in a 6-fold increase in this flavin radical signal, while the addition of MBQ resulted in the loss of the signal.

The rate of oxygen reduction is similar with CDH and CBQR and a comparison of the kinetics of glucose oxidase and CDH concluded that CDH reduces molecular oxygen by two electrons (*29*). Furthermore, the rate of the two-electron reduction of molecular oxygen to hydrogen peroxide by CDH was not increased by the addition of SOD (*16*). However, another laboratory reported the detection of free superoxide (*27*). For this manuscript, the reduction of oxygen was studied using ESR spin trapping techniques. Superoxide was not detected even when CDH levels were increased to concentrations as high as 2 mM. The superoxide−DMPO adduct spectrum was observed after the addition of ferric iron, Figure 7. This was detected as a hydroxyl radical−DMPO adduct because iron catalyzes the degradation of the superoxide−DMPO adduct to give the hydroxyl adduct (*27*). Upon the addition of 1 mM Desferal, no signal was obtained for any of the iron concentrations used in the analysis. Addition of catalase decreased the intensity of signals for

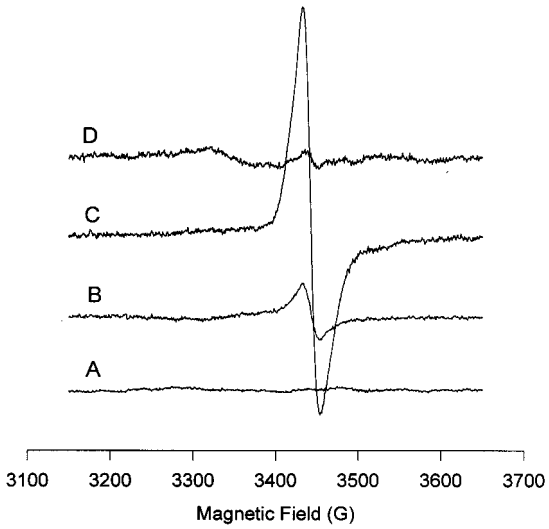

FIGURE 6: Cold temperature ESR spectra (8 K) of CBQR. Spectrum A was detected using 100 μM CBQR. Spectrum B was detected using 100 μM CBQR and 10 mM cellobiose. Spectra C and D were detected using similar conditions to spectrum B with the addition of 10 mM ferric chloride or 10 mM methyl-1,4-benzoquinone, respectively.

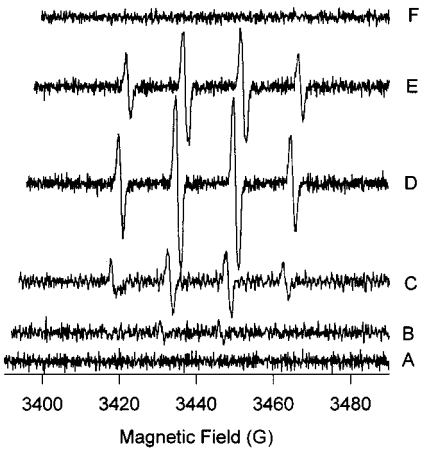

FIGURE 7: DMPO spin adduct ESR spectra detected upon incubation of CDH with cellobiose and various concentrations of iron. Spectrum A was detected in an aerobic reaction mixture containing 10 μM CDH, 1 mM cellobiose, 1 μM ferric chloride, 1 mM Desferal and 50 mM DMPO in 50 mM acetate buffer, pH 4.5. Spectrum B is the same as A without Desferal. Spectra C and D were detected using condition similar to B with the addition of 10 or 100 μM ferric chloride, respectively. Spectra E and F were detected using the same conditions as D with the addition of 1000 units of catalase or 1000 units of catalase and SOD, respectively.

the radicals in the reactions containing iron. The addition of both SOD and catalase completely abolished the signals at all iron concentrations. The data suggest that molecular oxygen acts as a two-electron acceptor with respect to CDH and that superoxide is produced through an iron mediated pathway.

DISCUSSION

Cellobiose dehydrogenase has two distinct domains, a 55 kDa C-terminal flavin domain that contains the site of cellobiose oxidation and a 35 kDa N-terminal heme domain that appears to be involved in the activation of the flavin by stabilizing the flavin radical. The heme appears to be located in proximity to the site of substrate reduction because of the observed alteration to the electronic environment of the heme upon the addition of the two-electron acceptor MBQ that was detected using cold temperature ESR and by the decrease in the activation energy for the one electron acceptor ferric iron. This leads us to also speculate that the site of substrate reduction in CDH lies in or around the cleft between the flavin and heme domains. The two domains are connected by a 15 amino acid linker, of which 12 are threonine or serine with one proline located in the middle of the linker that may form a hinge between the two domains. A similar linker sequence is present between the domains of CDH from *Trametes versicolor* (*30*).

Having the site of substrate reduction in the cleft between the two domains of CDH would explain the finding that substrate increased the rate of electron transfer between the flavin and heme. Second, this would explain why two electron acceptors as varied as MBQ and ferric iron could be both reduced by the flavin and affect the reduction rates of the flavin and heme cofactors. A nonspecific-binding site located in the cleft between the domains would accommodate a wide range of electron acceptors and would explain the change in the electronic environment observed by cold temperature ESR during the reduction of MBQ. There are, however, additional concerns or examples that might indicate that substrate reduction does not necessarily take place in the cleft between the domains such as the reduction of cytochrome *c*. Cytochrome *c* is too large to enter the cleft between the domains. Cytochrome *c* reduction is almost certainly due to long-range electron transfer. Additionally, there are other proteins where the rate of oxidation or reduction is altered by the binding of substrate, presumably due to a change in the three-dimensional structure upon substrate binding. However, we are unaware of any such proteins that are as nonspecific with respect to substrate as is CDH. An X-ray crystal structure would be helpful in determining the true site of substrate reduction. Alternatively, production of recombinant CDH and site-directed mutagenasis experiments should give insight into the importance of the linker between the domains and access to the cleft between the domains.

An increase in the rate of electron transfer between both cellobiose and the flavin and the flavin and heme of CDH upon the addition of ferric iron was detected using stopped-flow spectroscopy, while only an increase in the rate of flavin reduction was detected upon the addition of MBQ. The altered electron transfer rates may be due to some change occurring in the secondary or tertiary structure of the enzyme upon substrate binding or by the substrate serving as an electron transfer component between the flavin and heme, possibly altering the potential of the flavin or heme. It could be argued that the effect of ferric iron is an artifact caused by the ligation of iron to the nitrogen and carbonyl oxygen of oxidized FAD, thus altering the potential of the flavin. No such argument can be made for MBQ and induced changes upon substrate binding or a coupling of the flavin and heme appear to be the most likely hypotheses. Previously, there has been no data showing that CDH substrates are bound. The data presented in this paper shows that there is a binding site and that, upon binding, the structural or electronic characteristics of CDH are altered as seen by the increased rate of cellobiose oxidation and heme reduction.

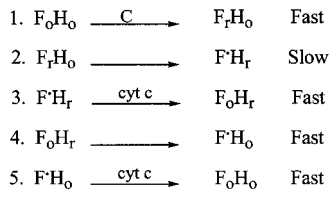

FIGURE 8: Redox state of the flavin and heme cofactors of CDH during the reaction of CDH, cellobiose and cytochrome *c*. Abbreviations: F_o, oxidized flavin; $F^{\bullet}$, flavin semiquinone radical; F_r, reduced flavin; H_o, oxidized heme; H_r, reduced heme; cyt c, cytochrome *c*; C, cellobiose. Fast and Slow relate to the relative pseudo-first-order rate constants of the reactions as compared to the rate of electron transfer between the fully reduced flavin and the oxidized heme of CDH at pH 6, reaction 2.

To study the reduction of cytochrome *c*, which was reported to be reduced at the heme domain (*25*, *31*), CDH was preincubated with cellobiose anaerobically in order to fully reduce the enzyme, resulting in a fully reduced ferrous heme and a fully reduced flavin. The rate of reduction of cytochrome *c* was found to be 0.7 s^{-1}, far lower than the rate of cytochrome *c* reduction under steady-state turnover, 16 s^{-1}. High concentrations of cellobiose were also found to inhibit cytochrome *c* reduction, presumably because the flavin was fully reduced under these high cellobiose conditions. This leads us to conclude that cytochrome *c* is reduced by the flavin radical and not by the reduced heme.

These results appear to contradict earlier studies, which concluded cytochrome *c* was reduced by the heme domain (*25*, *31*). This conclusion was based on pH studies and experiments where the pH was high and electron movement between the reduced flavin and the heme of CDH was not optimal. Rogers et al. (*22*) reported that during steady-state reduction of cytochrome *c* by CDH, the flavin was in its reduced form and the heme in its oxidized form, suggesting that the electron used to reduce cytochrome *c* was coming from the heme. Additionally, the rate of reduction of cytochrome *c* and the rate of electron transfer between the flavin and heme domains were found to be identical.

An alternative explanation is offered in Figure 8. Two electrons from cellobiose reduce the flavin of CDH, giving $FADH_2$ and ferric heme. One electron is then transferred from the $FADH_2$ to the heme yielding the flavin radical and reduced heme. The flavin radical of CDH then reduces cytochrome *c* resulting in the oxidized flavin and reduced heme. The remaining electron is passed from the reduced heme to the flavin resulting in a flavin radical and an oxidized heme. A second cytochrome *c* is reduced and the cycle is complete. At pH 6, the electron transfer between the flavin and the heme is very slow, 0.2 s^{-1}, by far the slowest reaction of those listed in Figure 8. Thus, the observed steady state intermediate at pH 6 is of reduced flavin and oxidized heme even though the flavin radical may actually be reducing cytochrome *c*.

The cold temperature ESR spectra of CDH and the independent heme domain were nearly identical. The *G* values for the oxidized heme group differ slightly, but not enough to indicate a spin interaction between the heme iron and the flavin or amino acids in the flavin domain. An interaction between the heme and the flavin domain may exist upon reduction as a result of structural changes within the two domains. Unfortunately, ferrous iron yields no ESR signal so this method cannot specifically address if an interaction between the two domains increases upon reduction.

The altered heme signal upon the addition of MBQ gave useful information. It is generally accepted that quinone reduction takes place at the flavin domain. The alteration in the electronic environment of the heme, as was seen by ESR, may be an indication that reduction takes place somewhere in the cleft between the heme and flavin domains. Unfortunately, a crystal structure for CDH is not available to verify the proximity of the flavin and heme to the cleft between the two domains.

The differences in the observed flavin radical ESR signals for CDH and CBQR upon addition of iron gives information as to the mechanism of one-electron reductions by CDH. The electron pair provided from cellobiose is split between the flavin and the heme of CDH. The electron passed to ferric iron comes from the more reactive flavin radical and thus quenches the flavin radical ESR signal. Conversely, reduction of iron by the independent flavin domain results in the formation of a flavin radical. This is the likely reason for the 38 kJ/mol higher activation energy for CBQR and is probably responsible for a large part of the 80% drop in the rate of iron reduction when the heme domain is cleaved from CDH.

The increased reactivity of the flavin radical of CDH might also increase the rate of electron passage from the flavin to such two electron acceptors as quinones. Stopped-flow experiments using MBQ showed that an electron was still passed to the heme and thus the flavin radical was formed. However, the time required to pass the second electron back from the heme group may negate any advantage gained from the activated flavin, thus explaining the similar reaction rates of CDH and CBQR for reduction of two electron acceptors.

The radical signal detected by ESR spectroscopy upon reduction of CBQR may be the result of residual iron contamination, even though attempts were made to rid the solution of contaminating iron by Chelex treatment. It may also be due to oxygen reduction. Oxygen is a triplet molecule and the direct reduction of oxygen by the singlet flavin is spin restricted. This may give rise to a flavin radical signal because of the time required to reverse the spin of the second electron so that it may be donated to oxygen. A third possibility is that an electron may be transiently located in the electron transfer route usually used for the transfer of electrons between the flavin and the heme. However, the inability of CBQR to reduce cytochrome *c* does not support the last hypothesis.

While published reports vary, our findings indicate that the reduction of oxygen occurs via a two-electron reduction to hydrogen peroxide without the release of superoxide. The superoxide−DMPO adduct was found only in samples that had small additions of iron. The observed ESR signal was generated from both iron-catalyzed superoxide and hydroxyl radical. Ferrous iron reduced molecular oxygen to generate superoxide, which at pH 4.5 dismutates at near maximal rates form hydrogen peroxide. The hydrogen peroxide reacts with additional ferrous iron to yield the hydroxyl radical. The decrease in radical signal intensity, which occurred upon the addition of catalase is due to the elimination of hydrogen peroxide and thus the elimination of the portion of the signal generated from hydroxyl radical. The remaining signal was the result of spin-trapped superoxide, which was verified by

the complete loss of signal when superoxide dismutase and catalase were added to the solution.

The loss of signal upon the addition of Desferal, which prevents the reduction of iron by CDH, is due to two factors. First, Desferal prevents hydrogen peroxide, generated directly by CDH reduction of molecular oxygen, from reacting to form the hydroxyl radical. Second, it prevents the production of superoxide from molecular oxygen and reduced iron. The complete loss of signal upon Desferal addition indicates the radical observed in the absence of Desferal was produced through the reaction of reduced iron and molecular oxygen, not through the release of superoxide by CDH, which should be unaffected by Desferal. Additionally, the finding that oxygen was slowly reduced to hydrogen peroxide (*16*) without the release of superoxide suggests some sort of binding in the reactive site of CDH.

CDH has been reported to reduce quinones, transition metal ions, molecular oxygen, and cytochrome *c*. The ESR and kinetic results from this study indicate that all four types of electron acceptors tested are reduced at the flavin domain of CDH. The rate of electron transfer between the flavin and heme in CDH ia greatly affected by the presence and nature of the electron acceptor. The steady state form of CDH during the reduction of two-electron acceptors such as quinones is different than with one-electron acceptors, such as iron. Production of superoxide was found to be caused by iron-mediated reduction of molecular oxygen. The results also suggest that substrate is bound at a reactive site and that this reactive site may be located in the cleft between the flavin and heme domains. Additional information about the binding sites and the interaction between the two domains would be benefited by a high-resolution crystal structure.

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