Cellulose Dehydrogenase from the Fungi *Phanerochaete chrysosporium* and *Humincola insolens*

**A FLAVOHEMOPROTEIN FROM HUMICOLA INSOLENS CONTAINS 6-HYDROXY-FAD AS THE DOMINANT ACTIVE COFACTOR**

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Cellulose dehydrogenases (CDH) were purified from cellulose-grown cultures of the fungi *Phanerochaete chrysosporium* and *Humincola insolens*. The pH optimum of the cellulose-cytochrome *c* oxidoreductase activity of *P. chrysosporium* CDH was acidic, whereas that of *H. insolens* CDH was neutral. The absorption spectra of the two CDHs showed them to be typical hemoproteins, but there was a small difference in the visible region. Limited proteolysis between the heme and flavin domains was performed to investigate the cofactors. There was no difference in absorption spectrum between the heme domains of *P. chrysosporium* and *H. insolens* CDHs. The midpoint potentials of heme at pH 7.0 were almost identical, and no difference in pH dependence was observed over the range of pH 3–9. The pH dependence of cellubiose oxidation by the flavin domains was similar to that of the native CDHs, indicating that the difference in the pH dependence of the catalytic activity between the two CDHs is because of the flavin domains. The absorption spectrum of the flavin domain from *H. insolens* CDH has absorbance maxima at 343 and 426 and a broad absorption peak at 660 nm, whereas that of *P. chrysosporium* CDH showed a normal flavoprotein spectrum. Flavin cofactors were extracted from the flavin domains and analyzed by high-performance liquid chromatography. The flavin cofactor from *H. insolens* was found to be a mixture of 60% 6-hydroxy-FAD and 40% FAD, whereas that from *P. chrysosporium* CDH was normal FAD. After reconstitution of the deflavoproteins it was found that flavin domains containing 6-hydroxy-FAD were clearly active but their cellulbiose oxidation rates were lower than those of flavin domains containing normal FAD. Reconstitution of flavin cofactor had no effect on the optimum pH. From these results, it is concluded that the pH dependence is not because of the flavin cofactor but is because of the protein molecule.

Cellulose, which consists of linear polymers of 1,4-linked β-D-glucose units, is the predominant structural component of plant cell walls and is estimated to account for about half of the organic material in the biosphere. Because of this abundance, cellulose biodegradation has a great impact on the global carbon cycle and recovery of natural resources. Cellulose biodegradation by filamentous fungi has generally been considered to involve only three types of hydrolytic enzymes, i.e. endo-1,4-β-D-glucanases, 1,4-β-D-glucobiohydrolases, and 1,4-β-D-glucosidase (1). Eriksson *et al.*, however, demonstrated that the degradation rate of cellulose by a cell-free culture of the white-rot fungus *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) was higher in the presence of oxygen than in its absence, suggesting the participation of some oxidation-reduction reaction (2).

In the course of cellulose degradation, many cellulolytic fungi produce extracellular cellulose-oxidizing enzymes as well as cellulose-hydrolyzing enzymes such as cellulases (3–5). Cellulbiose dehydrogenase (CDH, EC 1.1.99.18) is a flavohemoprotein that oxidizes cellulbiose using molecular oxygen as an electron acceptor (3). It was formerly known as cellulbiose oxidase (CBO, EC 1.1.3.25), but Fe(III)-containing compounds and quinones were found to have much higher affinity for this enzyme than does molecular oxygen (6, 7) so CBO was recently renamed CDH (8). Recent investigations have also demonstrated that another cellulbiose-oxidizing flavoprotein, cellobiosequinone oxidoreductase (CBQ, EC 1.1.5.1) (9, 10), is the flavin-containing domain of CDH produced by proteolytic activity (11–13). In addition to their catalytic function, both CDH and CBQ can bind to cellulose as well as many cellulases (11, 14). It was reported that the cellulose-binding site is located on the flavin domain but not in the catalytic site, because the enzyme bound to cellulose can still oxidize cellulbiose (14). Moreover, CDH adsorption on cellulose is also observed during cellulose degradation in vivo, especially at cracks on the cellulose surface formed by cellulases (15). Although the physiological function of this enzyme has not yet been clarified, there is no doubt that CDH contributes to cellulose biodegradation.

CDH oxidizes the reducing-end groups of cellulbiose, higher cellobiose, and cellulase, and even cellulase to the corresponding δ-lactones in the presence of electron acceptors (3, 16). Considering

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‡‡ The abbreviations used are: CDH, cellulbiose dehydrogenase; CBO, cellulbiose oxidase; CBQ, cellulbiose:quinone oxidoreductase; DCPIP, 2,6-dichlorophenol-indophenol; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
the cellobiose-oxidizing ability of both CDH and its flavin domain (CBQ), the flavin cofactor appears to be directly responsible for the oxidation of cellobiose (11). Concomitantly with cellobiose oxidation, CDH can reduce both Fe(III)-containing compounds and quinones effectively; however, the reduction processes are significantly different. The reduction rate of cytochrome c by CDH was reported to be extremely higher than that by the flavin domain, whereas the rates of DCPIP reduction by CDH and the flavin domain were similar (17). It has been reported that both cytochrome c and DCPIP were reduced by CDH at pH 4.2, whereas only DCPIP was reduced effectively at pH 5.9. This phenomenon was explained by stopped-flow kinetic study that both flavin and heme were reduced at a high rate at pH 4.2, whereas only flavin reduction proceeded quickly at pH 5.9 (18). From these observations, the reduction of cytochrome c is dependent on heme, and an electron is transferred from cellobiose to this electron acceptor via both FAD and heme, whereas the reduction of DCPIP is catalyzed only by flavin.

The thermophilic soft-rot fungus *Humicola insolens* also produces CDH under cellulolytic conditions, as do other cellulolytic fungi (19). However, this fungus produces CDH that has a neutral pH optimum in cytochrome c reduction, whereas CDH produced by other fungi have the optimum at acidic pH and show no activity at around neutral pH. In this study, therefore, CDH was purified from *P. chrysosporium* and from *H. insolens*, and the differences between two enzymes were investigated.

### EXPERIMENTAL PROCEDURES

#### Materials

*P. chrysosporium* CDH was purified from the medium of a cellulose-grown culture of *P. chrysosporium* as described by Samejima et al. (20). *H. insolens* CDH was purified from Celuzyme™ CASES (Novo Nordisk A/S, Bagsvaerd, Denmark) by using the same procedure. The purity of *P. chrysosporium* and *H. insolens* CDH preparations was confirmed by SDS-PAGE, isoelectric focusing, and the *R*<sub>c</sub> value (A<sub>421/A<sub>330</sub></sub>.

**n**-Cellobiose was purchased from ICN, 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (ubiquinone) and bovine heart cytochrome c were purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). Dithiothreitol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Papain was purchased from Elastin Products Co., Inc. (MO). FAD was purchased from Sigma. 6-Hydroxy-FAD was a generous gift from Dr. Vincent Massey, the University of Michigan. Calcium chloride (suprapure grade) was purchased from Merck (Darmstadt, Germany).

#### Methods

**Enzyme Assays**—Enzyme activities were assayed as described by Samejima and Eriksson (7). Ubiquinone (500 μM) reduction was monitored photometrically by following the absorbance at 406 nm (ε = 0.745 mM<sup>-1</sup> cm<sup>-1</sup>). Cytochrome c reduction was assayed by monitoring the increase of absorbance at 550 nm (ε = 22.4 mM<sup>-1</sup> cm<sup>-1</sup>). Spectrophotometric experiments were performed with a Shimadzu UV-1600PC spectrophotometer.

The following buffers (50 mM solutions) were used for the pH dependency studies of enzyme activity and midpoint potential of the heme domain: sodium citrate (pH 3.0–4.0), sodium acetate (pH 4.0–6.0), MES (pH 6.0, 6.5), HEPS (pH 7.0, 7.5), EPPS (pH 8.0, 8.5), and CHES (pH 9.0).

**SDS-PAGE and Isoelectric Focusing**—SDS-PAGE was carried out as described by Laemmli (21) using 10% polyacrylamide gel and a MiniPROTEAN<sup>®</sup> II Cell (Bio-Rad). Ultrathin-layer isoelectric focusing was described by Laemmli (21) using 10% polyacrylamide gel and a MiniIEF Cell (Bio-Rad).

**Limited Proteolysis and Isolation of the Flavin and Heme Domains**—Limited proteolysis of CDH by papain was performed essentially as described by Henriksson et al. (11). CDH (20 mg) was incubated in 25 ml of 20 mM phosphate buffer, pH 7.0, containing 2 mM dithiothreitol and 0.5 mg of papain for 6 h and then the solution was applied to a DEAE-Toyopearl 650S (Toyo Co. Ltd., Tokyo, Japan) column (11 × 120 mm) equilibrated with 20 mM phosphate buffer, pH 7.0, and eluted with a 150-ml linear gradient of KCl (0–250 mM) in the same buffer. The fractions containing the heme domain were concentrated by ultrafiltration (Ultrafree CL, Millipore Co.) and then used for experiments. The fractions containing the flavin domain, which had cellobiose-ubiquinone activity, were pooled and further purified by hydrophobic chromatography. The buffer solution was changed to 20 mM phosphate buffer containing 1 mM ammonium sulfate, pH 7.0, and then the flavin domain (pH 7.0) was applied to a cation-exchange column (DEAE-Toyopearl 650M; 120 × 1.5 cm) containing 1 M ammonium sulfate, pH 7.0. The column was washed with 10 ml of 10 mM sodium acetate, pH 7.0, and eluted with a 500-ml reverse gradient to 20 mM phosphate buffer. The ubiquinone-active fractions were pooled and concentrated by ultrafiltration as described above.

**Measurement of Midpoint Potential of the Heme Domain**—A direct electrochemical technique was used to measure the pH dependence of the oxidation reduction potential of the heme domain as described by Hagen (23). Glassy carbon, platinum, and Ag/AgCl were used as the working, counter, and reference electrodes, respectively. Cyclic voltammetry was performed in the presence of 50 mM MgCl<sub>2</sub>. The midpoint potential was determined by averaging the anodic and cathodic peak potentials.

**Identification of the Prosthetic Group of the Flavin Domain**—Flavin extraction from the flavin domain was based on the cold trichloroacetic acid method. That is, 100 μl of 50 mM flavin domain, cooled on ice, was mixed with an equal volume of ice-cold 20% trichloroacetic acid aqueous solution. The mixture was kept on ice for 10 min and centrifuged to remove the precipitate. The supernatant was neutralized by the addition of 1300 μl of 1 M phosphate buffer, pH 7.0. The samples were applied to Sep-Pak C-18 (Millipore Co., Ltd., Watertown, Massachusetts) containing 50% methanol, 10 mM sodium acetate buffer, pH 5.0, and concentrated by evaporation using a nitrogen gas stream. Flavin was analyzed by high-performance liquid chromatography with a JASCO-HPLC system (Jasco Co. Ltd., Tokyo, Japan) equipped with a Supelcosil LC-18-T column (4.6 × 250 mm; Supelco, Inc., PA) with a linear gradient of 20–30% methanol, 10 mM sodium acetate buffer, pH 5.0, and flavin was detected by measuring the absorption at 440 nm, which is the isosbestic point of the anionic and protonated forms of 6-hydroxy-FAD. Authentic FAD and 6-hydroxy-FAD were used as standards for identification and quantitation of flavin under the same conditions.

**Preparation of Deflavo-CDHs and Reconstitution with Flavins**—Preparation of deflavo-CDH was based on the method of Komai et al. (17). To obtain flavin domain containing 10 mM FAD and 6-hydroxy-FAD, deflavo-CDH was incubated with 2 mM flavin, FAD, or 6-hydroxy-FAD, and 5 mM dithiothreitol and 3.0 M (P. chrysosporium) or 2.5 M (H. insolens) CaCl<sub>2</sub> in 50 mM sodium acetate buffer, pH 4.0 (P. chrysosporium), or HEPES buffer, pH 7.5 (H. insolens), was kept on ice for 60 min, and then the solution was passed through a desalting column (Amupole SA; Tosoh Co. Ltd., Tokyo, Japan) equilibrated with 50 mM HEPES buffer, pH 7.5, to stop the reaction and to remove low molecular weight materials. The obtained deflavo-CDH (1 μM) was kept on ice. For reconstitution, deflavo-CDH was incubated with 2 mM flavin, FAD, or 6-hydroxy-FAD, and 5 mM dithiothreitol in 50 mM sodium acetate buffer, pH 4.0 (P. chrysosporium) or HEPES buffer, pH 7.5 (H. insolens) on ice for 60 min. The reconstituted preparations were concentrated by ultrafiltration as described above to remove excess free flavin and dissolved in the same buffers. The flavin domain was prepared by limited proteolysis as described in a previous section. For spectrophotometric determination, the deflavo-flavin domain was prepared by the same procedure but without flavin reconstitution. Native, deflavo-, and reconstituted flavin domains showed the same molecular weight on SDS-PAGE, indicating that the treatments had no effect on the proteolytic site.

**RESULTS**

**Purification, Characterization, and Limited Proteolysis of CDH**—CDH purified from culture solution of *P. chrysosporium* gave a single band at 90 kDa on SDS-PAGE (Fig. 1, lane 1), the isoelectric point was 4.2, and the *R*<sub>c</sub> value (A<sub>421/A<sub>330</sub></sub> was 0.62. During purification, it was found that crude cellulase powder from *H. insolens* contained three CDH fractions: one major fraction (94 kDa, pI 4.4, and R<sub>c</sub> 0.63) and two minor fractions (both 92 kDa, pI 4.0, and R<sub>c</sub> 0.63). Although all CDH was eluted in the same fraction in the first strong anion-exchanging chromatography (QAE-Toyopearl 550C), it was separated into two fractions, 94-kDa fraction and a 92-kDa fraction (resembling cellobiose dehydrogenase (DEAE-Toyopearl 650S) as reported by Schou et al. (19). However, the 92-kDa fraction was further separated into two minor CDHs by hydrophobic chromatography (phenyl-Toyopearl 650M). In this study, the major fraction of CDH (94 kDa) was used for further investigation as CDH from *H. insolens*.
that of domains but the cleavage site was somewhat different from proteolysis occurred similarly between the heme and flavin domains from masses 25 and 70 kDa were the heme- and flavin-containing (17). On the other hand, the bands corresponding to molecular respectively, in accordance with the results reported by Henriksson trials of the CDHs. From the absorption spectra of each band, as described under “Experimental Procedures” we were loaded on 10% polyacrylamide gel.

The CDHs from P. chrysosporium and H. insolens had different values of pH optimum when the pH dependence of cytochrome c reducing activity was monitored using cellobiose as a substrate (Fig. 2); CDH from H. insolens had the pH optimum around pH 7.5–8.0, whereas CDH from P. chrysosporium had the optimum at pH 3.5–4.0. The specific activity of H. insolens CDH at pH 7.5 was 3.3 s⁻¹ and was 10-fold lower than that of P. chrysosporium CDH at pH 4.0 (37 s⁻¹). Absorption spectra of the oxidized CDHs are shown in Fig. 3. Both enzymes showed typical hemoprotein spectra having an absorption maximum at 421 nm (γ band). It should be noted, however, that minor differences in the region of 450–500 and 300–400 nm were found between the two CDHs because of the difference in the flavin chromophore, as described below. The spectral patterns of the two minor fractions of CDH from H. insolens described above were similar to that of the major fraction (data not shown), suggesting that all CDHs from H. insolens have the same flavin chromophore. To clarify the reason for the spectral difference, limited proteolysis of the two CDHs with papain was carried out to isolate the heme and flavin domains for further investigation. As shown in Fig. 1, two bands were isolated and purified from each of the CDHs. From the absorption spectra of each band, as described in the following sections, it was concluded that the bands corresponding to molecular masses 35 and 55 kDa were the heme and flavin domains of P. chrysosporium CDH, respectively, in accordance with the results reported by Henriksson (17). On the other hand, the bands corresponding to molecular masses 25 and 70 kDa were the heme- and flavin-containing domains from H. insolens CDH, respectively, indicating that proteolysis occurred similarly between the heme and flavin domains but the cleavage site was somewhat different from that of P. chrysosporium CDH.

Absorption Spectrum and Midpoint Potential Comparison of Heme Domains—The absorption spectra of the oxidized heme domains separated from P. chrysosporium and H. insolens CDH are shown in Fig. 4. No particular difference was observed in the oxidized heme spectra of the two CDHs. Moreover, no difference in the reduced spectra obtained by addition of sodium dithionite was observed either (data not shown). No significant difference in the redox properties of the heme domains was revealed by determination of the pH dependence of the midpoint potential of each heme domain using a direct electrochemical technique, as shown in Fig. 5. The potentials of the P. chrysosporium and H. insolens heme domains were 190 and 185 mV at pH 3.0, respectively, then declined until around pH 4.0–5.0, and became pH-independent over pH 6.0. The potentials of the P. chrysosporium and H. insolens heme domains at pH 7.0 were 130 and 126 mV, respectively. The value for the P. chrysosporium enzyme at pH 4.0 is 180 mV, which is in good agreement with the previously published value of 164 mV obtained by a dye-mediated optical redox titration (6). Characterization and Comparison of the Flavin Domains—The pH dependence of the cellobiose-oxidizing activity of CDH and the flavin domain was monitored in terms of the reduction rate of ubiquinone (Fig. 6). As in the case of P. chrysosporium,
the flavin domain of *H. insolens* CDH had cellobiose-oxidizing activity at almost the same level as that of intact CDH, whereas the heme domains of *P. chrysosporium* and *H. insolens* CDH had no activity. Thus, it is concluded that the catalytic site of cellobiose oxidation is in the flavin domain of each CDH, and the difference in pH dependence of CDH activity is solely because of the flavin domains.

In contrast to the heme domains, the absorption spectra of the flavin domains were considerably different between the two CDHs (Fig. 7). The flavin domain from *P. chrysosporium* showed yellow coloration and had absorption maxima at 387 and 457 nm, typical of flavin (Fig. 7, inset). On the other hand, the flavin domain from *H. insolens*, showed a green coloration with absorption maxima at 343 and 426, resembling the spectrum of 6-hydroxyflavin, but a broad peak was observed at around 660 nm at pH 7.5. The long-wavelength absorption peak disappeared at pH 5.0, and the color changed to yellow, consisting with 6-hydroxyflavin derivatives. The long-wavelength absorption was shifted to 660 nm in the flavin domain of *H. insolens* CDH (Fig. 7), as compared with the broad peak at 600 nm of free 6-hydroxy-FAD (Fig. 7, inset).

**Identification of the Prosthetic Group of the Flavin Domain**—The prosthetic group of the flavin domain, extracted by treatment with trichloroacetic acid, was analyzed by HPLC (Fig. 8). The isolated flavin was non-fluorescent even after phosphodiesterase treatment. A comparison of the retention time with that of authentic FAD indicated that the flavin domain from *P. chrysosporium* contained at least 95% FAD, whereas the *H. insolens* flavin domain contained a mixture of 60% 6-hydroxy-FAD and 40% FAD. After treatment of authentic FAD and 6-hydroxy-FAD with phosphodiesterase, the samples were subjected to HPLC. The retention times of the phosphodiesterase-treated authentic FAD and 6-hydroxy-FAD were shifted from and to 8.5 and 15.5 min, respectively, because of the conversion from FAD- to FMN-type compounds. When the flavin cofactors from CDHs were also treated with phosphodiesterase, their retention times were identical with those of the authentic FMNs. Thus, it was concluded that the flavin cofactors from CDHs were FAD forms and that 6-hydroxy-FAD was the dominant cofactor of *H. insolens* CDH.

**Preparation of Deflavo-enzymes and Reconstitution with Flavins**—To examine the effect of 6-hydroxy-FAD on the activity of the flavin domain, the deflavo-domains from the two CDHs were prepared, and their cellobiose-oxidizing activity was determined after reconstitution with FAD or 6-hydroxy-FAD. The use of the flavin domains prepared after limited proteolysis was unsuccessful. However, active flavin domains were successfully obtained by reconstitution of whole deflavo-CDHs with flavin followed by limited proteolysis of the reconstituted CDHs. The deflavo-CDHs from *P. chrysosporium* and *H. insolens* were prepared by incubation of the enzyme with a high concentration of CaCl₂ in the presence of cellobiose under the different conditions, as described under “Experimental Procedures.”

The absorption spectra of the deflavo and reconstituted flavin domains are shown in Fig. 9. The absorption spectrum of the domain from *P. chrysosporium* CDH reconstituted with FAD (Fig. 9A) was almost identical with that of the native domain, whereas that of the domain from *H. insolens* CDH reconstituted with 6-hydroxy-FAD CDH (Fig. 9B) was similar to but not identical with that of native domain, which contains a mixture of FAD and 6-hydroxy-FAD, as shown in the previous section. Compared with the spectrum of the native flavin domain (Fig. 7A), reconstitution with 6-hydroxy-FAD caused loss of the shoulder peak around 470 nm, but the long-wavelength absorbance peak of 6-hydroxy-FAD shifted to 660 nm after binding to the protein, as in the native sample.

Cellobiose-oxidizing activity of the enzymes with reconstituted FAD or 6-hydroxy-FAD is shown in Table I. The activity of the *P. chrysosporium* domain reconstituted with FAD was comparable with that of the native sample, indicating that the reconstitution procedure had been successful. The activity of the flavin domains reconstituted with 6-hydroxy-FAD from *P. chrysosporium* and *H. insolens* amounted to about one-third at
pH 4.0 and less than one-sixth at pH 7.5 as compared with that of the domains reconstituted with FAD. The activity of the \textit{H. insolens} enzyme reconstituted with FAD was 1.8-fold higher than that of the native enzyme at pH 7.5, whereas that of the enzyme reconstituted with 6-hydroxy-FAD was about 60% of the native one, a finding which can be well explained by the fact that the native enzyme contains a mixture of 60% 6-hydroxy-FAD and 40% FAD.

Thus, these spectrophotometric and activity measurements of the reconstituted enzyme confirmed that CDH from \textit{H. insolens} contains 6-hydroxy-FAD as the dominant cofactor that is catalytically active, though less than FAD. It is also clear that the pH dependence of the activity was not because of the flavin cofactor but, rather, was because of the protein molecule because the pH dependence of the two CDHs was little affected by flavin replacement, as judged from the activities at pH 4.0 and pH 7.5.

DISCUSSION

Three CDH fractions were isolated from \textit{H. insolens} crude cellulase, whereas only one CDH was contained in culture solution of \textit{P. chrysosporium}. Two fractions containing 92 and 94 kDa species were separated from \textit{H. insolens} by anion exchange chromatography, as reported by Schou \textit{et al.} (19), but one of them (92 kDa) was further separated into two fractions by hydrophobic chromatography using phenyl-Toyopearl 650M in this experiment although these two minor CDH species had the same molecular mass (92 kDa), the same isoelectric point (4.0), and similar absorption spectra. It is not clear at the moment whether these fractions are the different gene products or products of post-translational modification. In this study, the major fraction of CDH (94 kDa, pI 4.4) was used for experiments as CDH from \textit{H. insolens}.

The optimum pH of the cellobiose-cytochrome \textit{c} oxidoreductase activity of CDH from \textit{P. chrysosporium} was in the acidic pH range, whereas that of CDH from \textit{H. insolens} was at neutral pH. This is consistent with the pH optima for cellulase activity and fungal growth on cellulose, which are pH 3–5 and 7–8 for \textit{P. chrysosporium} and \textit{H. insolens}, respectively (25, 26). In this way CDH can cooperate effectively with cellulase in the cellulose biodegradation process in both fungi. The absorption spectrum of the oxidized forms of two CDHs were typical of heme proteins though there were small differences between two spectra. To clarify these phenomena, limited proteolysis with papain was performed to prepare heme- and flavin-containing domains. Like \textit{P. chrysosporium} CDH (11), \textit{H. insolens} CDH was cleaved into two domains by papain. In both CDHs, the sum of the molecular weights of the two domains was almost identical to the molecular weight of the native CDH, suggesting that papain cleaved a single site between the two domains, although the cleavage site in \textit{H. insolens} CDH was different from that in \textit{P. chrysosporium} CDH, resulting in a smaller molecular weight of the heme domain and a larger molecular weight of the flavin domain.
The absorption spectrum and midpoint potential were measured to characterize the heme domain. Both heme domains showed typical b-type heme spectra, and no difference was found in either the oxidized or the reduced spectra. Moreover, they showed almost the same pH dependence of midpoint potential. Although Schou et al. suggested that a difference in the environment of heme causes the difference of optimum pH between P. chrysosporium and H. insolens CDH (19), the present experiments clearly demonstrate that the difference in pH dependence is not because of heme as a prosthetic group but rather is because of the flavin domain. The profile of pH dependence of cellobiose-oxidizing activity was very similar for the flavin domain and intact CDH in both cases, indicating that the optimum pH of CDH mostly depends on the activity of the flavin domain.

The prepared flavin domain of H. insolens CDH showed a green color with a broad peak at long wavelength, whereas that of P. chrysosporium CDH was yellow, showing a typical riboflavin-type spectrum. A similar spectrum to that of the flavin domain of H. insolens CDH has been reported by Mayhew et al. for the green chromophore, 6-hydroxy-FAD, in electron-transferring flavoprotein (ETF) from Peptostreptococcus elsdienii (27). Upon binding to the CDH flavin domain, free 6-hydroxy-FAD showed a shift of the long-wavelength absorption from 600 to 660 nm, as in the case of ETF. HPLC analysis of extracted flavin was carried out to identify and quantify the prosthetic group of the flavin domain. Comparison with authentic samples indicated that the flavin cofactor of H. insolens CDH is a mixture of 60% 6-hydroxy-FAD and 40% FAD, whereas that of P. chrysosporium CDH consisted only of FAD. This finding accounts well for the existence of shoulders at 380 and 475 nm in the absorption spectrum of H. insolens flavin domain (Fig. 7A). Morpeth and Jones isolated cellobiose quinone dehydrogenase (CBQ) as a simple flavoprotein from P. chrysosporium and showed that it has a nonfluorescent green chromophore as the active cofactor (28). The spectrum of their native enzyme suggested that 6-hydroxy-FAD was present as a minor component of the prosthetic group. However, in the case of H. insolens CDH, 6-hydroxy-FAD was contained as the dominant cofactor. The spectral patterns of the other minor fractions of CDH from H. insolens were similar to that of the major fraction, suggesting that all CDHs from H. insolens have the 6-hydroxy flavin chromophore. Thus, H. insolens CDH is unique in containing 6-hydroxy-FAD as the dominant cofactor. As the activity of CDH containing 6-hydroxy-FAD was lower than that of the enzyme reconstituted with normal FAD, the physiological significance of the presence of 6-hydroxy-FAD is not clear but may reflect biosynthetic factors.

From the reconstitution experiments with 6-hydroxy-FAD or FAD, it is clear that the pH dependence was not because of the flavin cofactor but was intrinsic to the protein molecule. Reconstitution with FAD enhanced the cellobiose-oxidizing activity of H. insolens flavin domain, indicating that CDH containing 6-hydroxy-FAD is active but has a lower cellobiose-oxidizing activity than the enzyme containing normal flavin. Systematic studies using various modified flavins with different redox potentials will be needed to see whether potential change of the prosthetic group affects the activity because the midpoint potential of FAD (−219 mV; Ref. 29) is more positive than that of 6-hydroxy-FAD (−255 mV; Ref. 30), and such a study is in progress.

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![Fig. 9. Absorption spectra of oxidized and reduced flavin domains reconstituted with FAD or 6-hydroxy-FAD and their deflav flavin-type spectrum.](image-url)
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