Role of the Flavin Domain Residues, His689 and Asn732, in the Catalytic Mechanism of Cellobiose Dehydrogenase from Phanerochaete Chrysosporium[†]

Frederik A. J. Rotsaert, V. Renganathan, and Michael H. Gold*

Department of Biochemistry and Molecular Biology, OGI School of Science and Engineering, Oregon Health and Science University, Beaverton, Oregon 97006-8921

Received October 31, 2002; Revised Manuscript Received February 20, 2003

ABSTRACT: Cellobiose dehydrogenase is an extracellular flavocytochrome, which catalyzes the oxidation of cellobiose and other soluble oligosaccharides to their respective lactones, while reducing various one- and two-electron acceptors. Two residues at the active site of the flavin domain, His689 and Asn732, have been proposed to play critical roles in the oxidation of the substrate. To test these proposals, each residue was substituted with either a Gln, Asn, Glu, Asp, Val, Ala, and/or a His residue by site-directed mutagenesis, using a homologous expression system previously developed in our laboratory. This enabled an examination of the functional, stereochemical, and electrostatic constraints for binding and oxidation of the substrate. The steady-state kinetic parameters for the variant proteins were compared using cellobiose and its epimer, lactose, as the substrates. The H689 variants all exhibit >1000-fold lower k_{cat} values, while the K_m values for both substrates in these variants are similar to that of the wild-type enzyme. This supports the proposed role of this His residue as a general base in catalysis. The N732 variants exhibit a range of kinetic parameters: the k_{cat} values for oxidation are 5−4000-fold lower than that for the wild-type enzyme, while the K_m values vary between similar to and 60-fold higher than that for the wild-type. The difference in binding energy between cellobiose and lactose was calculated using the relationship $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{lactose}/(k_{cat}/K_m)_{cellobiose}]$. This calculation for the wild-type enzyme suggests that lactose binds considerably more weakly than cellobiose (7.2 kJ/mol difference), which corresponds to one extra (cumulative) hydrogen bond for cellobiose over lactose. Mutations at Asn732 result in a further weakening of lactose binding over cellobiose (2−4 kJ/mol difference). The results support a role for Asn732 in the binding of the substrate.

Cellobiose dehydrogenase (CDH)[1] is secreted by various cellulolytic fungi (*1*). As for cellulases, the expression of CDH is promoted when cells are grown in the presence of cellulose and repressed in the presence of glucose (*2*). The role of CDH in wood degradation is still under consideration, but evidence suggests the enzyme is involved in cellulose degradation and may be involved in lignin degradation (*1*). Recently, a mutant strain of *Trametes versicolor*, which is incapable of producing CDH under normal induction conditions, was described (*3*). The mutant strain exhibited a decreased ability to grow on birch wood and on plates with crystalline cellulose as the carbon source, suggesting that CDH may be essential for growth on wood and cellulosic substrates.

To our knowledge, CDH is the only extracellular flavocytochrome reported, and it has been purified from several filamentous fungi (*1*) of which the enzyme from *Phanerochaete chrysosporium* is the best studied (*4*, *5*). Two cofactors, flavin adenine dinucleotide (FAD) and protoporphyrin IX, are noncovalently bound to two distinct domains of the single polypeptide chain, and the two domains are connected by a heavily glycosylated, hydroxyamino-rich linker peptide (*2*, *6*, *7*), similar to that found in other modular cellulolytic enzymes (*8*). The flavin domain is involved in the oxidation of cellobiose and other soluble oligosaccharides, such as cellotetraose and lactose, to their corresponding lactones (*9*, *10*). The 1β-hydroxyl of the reducing end of the substrate is oxidized (*11*) with concomitant reduction of FAD, and the electrons are subsequently transferred directly to two-electron acceptors such as 2,6-dichlorophenolindophenol (DCPIP), or via the heme domain, to one-electron acceptors such as cytochrome *c* (*1*).

Recently, crystal structures were reported for each of the two separate domains of CDH, the heme domain (CDHcyt) (*12*), and the flavin domain (CDHdh) (*13*). CDHcyt has a unique fold, resembling that of the antibody Fab V_H domain, and the heme iron is ligated by residues Met65 and His163 (*10*, *12*). On the basis of gene sequence comparisons, CDHdh was predicted to be a member of the glucose-methanol-choline (GMC) family (*2*, *14*), which includes methanol oxidase, cholesterol oxidase (CHO), glucose oxidase (GOX), and choline dehydrogenase (*15*, *16*). These FAD-dependent oxidoreductases catalyze the oxidation of nonactivated al-

[†] This research is supported by Grant DE-FG03-99ER20320 (to M.H.G.) from the U.S. Department of Energy, Division of Energy Biosciences.

* To whom correspondence should be addressed. Telephone: (503) 646-0957. Fax: (503) 748-1464. E-mail: mhgold@myexcel.com.

[1] Abbreviations: CDH, cellobiose dehydrogenase; CDHcyt, heme domain of CDH; CDHdh, flavin domain of CDH; CHO, cholesterol oxidase; DCPIP, 2,6-dichlorophenol-indophenol; DTT, dithiothreitol; GMC, glucose-methanol-choline; GOX, glucose oxidase; rCDH, recombinant wild-type CDH; TCA, trichloroacetic acid.

10.1021/bi027092k CCC: $25.00
Published on Web 03/22/2003

cohols, and their main consensus sequences include the FAD binding domain. While GMC enzymes catalyze the oxidation of different substrates and exhibit little sequence similarity in their substrate-binding domains, the tertiary structures of CDHdh (*13*), GOX (*15*, *17*), and CHO (*18*, *19*) reveal a highly conserved catalytic site, containing two conserved residues, a His and an Asn or His, which are located in the structurally conserved C-terminal region. In the CDHdh crystal structure, these residues, His689 and Asn732, are positioned near the isoalloxazine ring. Conservation of the position of these residues in GMC proteins suggests a similar activation mechanism for oxidation of the substrate.

Molecular modeling has identified several possible interactions between cellobiose and protein, which provide insights into catalysis and substrate binding (*13*). The channel from the surface to the catalytic site accommodates a cellobiose molecule, and an active-site water is replaced by the anomeric β-hydroxyl CO1. In addition, the anomeric α-hydrogen CH1 of cellobiose is oriented toward the FAD-N5. His689 is the only basic residue near the β-hydroxyl of cellobiose, and the orientation of His689 suggests that it may act as a general base. Hydrogen bonding of Asn732 with the anomeric hydroxyl suggests that this residue may contribute to binding of the substrate.

In this study, we assess of the roles of His689 and Asn732 in binding and oxidation of the substrates, cellobiose and lactose, using site-directed mutagenesis and steady-state kinetic measurements. In addition, we investigate the effect of the mutations on the electronic properties of the flavin cofactor.

EXPERIMENTAL PROCEDURES

Organism. Growth and maintenance of the *P. chrysosporium* auxotrophic strain OGC316-7 (Ura11) and prototrophic transformants were as described (*20*). *Escherichia coli* DH5α was used for subcloning plasmids.

Construction of the Mutant Plasmids. The site-directed mutations at the Asn732 position (N732H/Q/A/E/D) were introduced into pUGC1, which contains 1.1 kb of the *gpd* gene promotor, fused to the *P. chrysosporium cdh-1* coding region (3.1 kb) (*10*, *21*), using the Transform site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA) (*10*). A mutagenic primer introduced the N732 mutations, changing the AAC codon (Asn) to CAC (His), CAG (Gln), GCT (Ala), GAG (Glu), or GAC (Asp). The mutant plasmids were isolated, and the mutation was confirmed by dideoxy sequencing (Oregon National Primate Research Center, Beaverton, OR). A protocol, similar to that described above, was used to create site-directed mutations at the His689 position (H689N/Q/A/E/V).

DNA Transformation of P. chrysosporium. Protoplasts of *P. chrysosporium* OGC316-7 (Ura11) were prepared as described (*20*, *22*) and transformed with EcoRI-linearized mutant plasmids (2 μg), and potential transformants were screened for uracil prototrophy (*20*, *22*). Subsequently, conidia from prototrophs were transferred to high carbon-high nitrogen stationary liquid cultures, containing 2% glucose, Kirk's salts, 20 mM sodium-2,2-dimethylsuccinate, and 12 mM ammonium tartrate as described (*10*, *21*). Production of CDH was assayed using the cytochrome *c* and DCPIP assays (*10*) and by Western blot analysis of extracellular protein using polyclonal antibodies raised against *P. chrysosporium* CDH (*21*). The transformed strains exhibiting the highest activity and/or highest CDH protein levels were purified by isolating single basidiospores as described (*23*, *24*), and the progeny were rescreened for CDH activity and/or CDH secretion.

Production and Purification of Recombinant Wild-Type and Variant Proteins. Each recombinant strain was grown from a conidial inoculum in high carbon-high nitrogen stationary liquid culture, and the variant proteins were purified from the extracellular medium on day 7 (*10*). The extracellular fluid was concentrated and dialyzed against 20 mM potassium phosphate, pH 6. Subsequently, the variant proteins were purified by cellulose-affinity chromatography and gel filtration (Sephacryl S200 HR) (*10*).

Flavin Cofactor Extraction. The free flavin cofactor was obtained by the cold trichloroacetic acid (TCA) method (*25*, *26*). To 20−50 μM recombinant wild-type CDH (rCDH) or flavin domain variants (100 μL) was added 20% ice-cold TCA (100 μL), and the mixture was incubated on ice for 10 min. The precipitate was removed by centrifugation, and the supernatant was neutralized by the addition of 600 μL of Tris-HCl (1 M), pH 8.0. The concentration of extracted FAD was calculated from a standard curve, using the absorbance at 450 nm. The concentrations of the proteins prior to extraction were calculated using a Soret absorbance at 421 nm ($\epsilon_{421} = 100$ mM^{-1} cm^{-1}).

Preparation of Truncated Flavin Domains. The flavin domains of recombinant wild-type and variant proteins were prepared, using limited proteolysis (*7*, *27*). The flavocytochromes (20 μM), purified to homogeneity or purified through the cellulose affinity step, were incubated with 200 μg/mL of papain (Boehringer-Mannheim GmbH, Germany) in 0.1 M potassium phosphate, pH 7, containing 2 mM EDTA and 2 mM dithiothreitol (DTT), for 2 h at room temperature. This digested protein solution was concentrated and dialyzed against 10 mM Tris-HCl, pH 8, and the flavin domain was purified by FPLC, using a mono-Q column with a 0−300 mM NaCl gradient in 10 mM Tris-HCl, pH 8.

SDS−PAGE and Immuno (Western) Blot Analysis. SDS−PAGE was performed using a 12% Tris-glycine system (*28*) in a Miniprotean II apparatus (Bio-Rad, Hercules, CA), and gels were stained with Coomassie blue. Proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore) using a Bio-Rad system apparatus. Immunodetection was performed with the Western-Light chemiluminescent detection system (Tropix, Bedford, MA), using polyclonal antibodies raised against *P. chrysosporium* CDH (*2*).

Spectroscopic Procedures. Electronic absorption spectra of rCDH as well as N732 and H689 variants were obtained at room temperature in 20 mM Na succinate, pH 4.5, with a Cary 100 spectrophotometer (Varian, Australia). The proteins were reduced with either cellobiose (500 μM) or sodium-dithionite.

Enzyme Assays and Kinetic Procedures. CDH activity was measured using either the cytochrome *c* or the DCPIP assay at room temperature (23 ± 1 °C) (*10*). The specific activity of cellobiose oxidation with DCPIP as electron acceptor was determined with 400 μM cellobiose and 35 μM DCPIP in 20 mM sodium-succinate, pH 4.5 [$\epsilon_{515} = 6.8$ mM^{-1} cm^{-1}]. The steady-state kinetic parameters for cellobiose oxidation

were also determined by monitoring ferrocytochrome *c* formation [$\Delta\epsilon_{red-ox}$ (550 nm) = 21 mM^{-1} cm^{-1}] (*29*). The assays contained a fixed amount of ferricytochrome *c* (12.5 μM) and varying levels of cellobiose (5−800 μM) or lactose (50−60 mM) in 20 mM sodium-succinate, pH 4.5. Stock solutions of cellobiose and lactose were left overnight for equilibration (mutarotation). A nonlinear regression algorithm was employed to obtain the Michaelis−Menten kinetic parameters k_{cat} and K_m, using a publicly available software package, LSW-Data analysis Toolbox (MDL Information System Inc.), integrated in Microsoft Excel.

pH Dependence of Cellobiose Oxidation. The effect of pH on CDH activity was monitored in the presence of cellobiose (400 μM) and the electron acceptors cytochrome *c* (12.5 μM) or DCPIP (35 μM). The buffer systems (20 mM) used were sodium-succinate (pH 3−5), potassium-phosphate (pH 5.5−7.5), and Tris (pH 8.0−9.0). Cytochrome *c* reduction was measured at 550 nm. The spectral characteristics of DCPIP are dependent on the pH of the reaction medium. The following wavelength and extinction coefficients were used: pH 3.0−4.5, 515 nm (6.8 mM^{-1} cm^{-1}); pH 5.0, 535 nm (6.8 mM^{-1} cm^{-1}); pH 5.5, 585 nm (8.2 mM^{-1} cm^{-1}); pH 6.0, 600 nm (12.7 mM^{-1} cm^{-1}); pH 6.5, 600 nm (15.7 mM^{-1} cm^{-1}); pH 7, 600 nm (16.8 mM^{-1} cm^{-1}); and pH 7.5−9.0, 600 nm (17.7 mM^{-1} cm^{-1}).

Chemicals. Glucose and potassium-phosphate were obtained from J. T. Baker (Phillipsburg, NJ). Molecular biology reagents were obtained from New England Biolabs (Beverly, MA) or Stratagene (La Jolla, CA). All other chemicals were purchased from Sigma. Solutions were prepared in Millipore Q-50 (Millipore Corp., Bedford, MA) purified water.

RESULTS

Preparation of the Cellobiose Dehydrogenase N732 and H689 Variants. A variety of substitution mutations at the H689 and N732 positions were introduced into pUGC1 (*10*), and the mutations were confirmed by DNA sequencing. The mutant CDH enzymes were homologously expressed in *P. chrysosporium* (*21*). Western blot analysis was utilized to select strains secreting the highest level of mutant protein because CDH activity in most of these mutant transformation cultures was extremely low. The CDH variants were detected by electronic absorption spectroscopy in the concentrated extracellular fluid. The variant proteins were purified to homogeneity, based on their R_z values (A_{421}/A_{280} > 0.6). On the basis of the amount of CDH protein purified from the extracellular medium, the expression levels of the flavin domain variants were 20−100% of that observed for rCDH. SDS−PAGE analysis indicated a molecular mass of 90 kDa for all the purified flavin domain variants, similar to wild-type CDH (*21*). FAD cofactors were isolated from rCDH and the variant proteins by the TCA precipitation method (*25*, *26*). Their electronic absorption spectra were similar to authentic FAD, exhibiting maxima at 380 and 450 nm. The flavin occupancy was ∼90−95% for rCDH and the flavin domain variants.

Catalytic Properties of H689 Variants. The CDH activity in the extracellular media of the H689 variants was drastically reduced as measured by the cytochrome *c* and DCPIP assays. Despite the very low residual activities, a combination of a much larger amount of enzyme used in the assays (100−500 nM as compared to 1−20 nM), a more sensitive UV−vis spectrophotometer (Cary 100), and longer recording times allowed us to conduct a steady-state kinetic analysis of the H689 variants with cytochrome *c* as the electron acceptor (Table 1). The k_{cat} for cellobiose oxidation ranged from 0.002 to 0.009 s^{-1}, a ∼5000-fold decrease from rCDH. However, each variant exhibited a K_m similar to the wild-type enzyme (20 μM). The steady-state kinetic parameters were also determined for a second substrate, lactose (Table 1). rCDH and the flavin domain variants H689A and H689Q exhibited k_{cat} values for lactose oxidation that were similar to that for cellobiose oxidation. However, the K_m values for lactose, with both rCDH and the H689 variants, were 15-fold higher than those for cellobiose.

Table 1: Steady-State Kinetic Parameters for rCDH and H689 Variants[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)
Cellobiose oxidation[b]		
rCDH	15.5	16
H689N	0.004	19
H689Q	0.004	21
H689A	0.003	21
H689V	0.002	31
H689E	0.009	22
Lactose oxidation[b]		
rCDH	14.3	270
H689Q	0.003	290
H689A	0.003	280

[a] Reactions were performed at room temperature in 20 mM Na-succinate, pH 4.5. K_m and k_{cat} for cellobiose and lactose were determined using 12.5 μM cytochrome *c* and varying the substrate concentration between 5 and 400 μM (cellobiose) and between 50 μM and 2 mM (lactose). [b] Variance was less than 5% of the values presented.

Table 2: Steady-State Kinetic Parameters for rCDH and the N732 Variant[a]

	Cellobiose oxidation[b]		Lactose oxidation[b]		
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	$\Delta(\Delta G)$[c] (kJ mol^{-1})
rCDH	15.5	16	14.3	270	7.2
N732H	2.7	40	1.7	2300	11.2
N732Q	1.0	41	1.2	4000	10.9
N732A	0.4	17	0.7	1200	9.0
N732E	0.21	230	0.3	16 600	9.4
N732D	0.004	170	0.005	11 400	9.2

[a] Reactions were performed in 20 mM Na-succinate, pH 4.5, at room temperature. K_m and k_{cat} for cellobiose were determined, using 12.5 μM cytochrome *c* and varying the substrate concentration between 5 and 800 μM (cellobiose) and between 50 μM and 60 mM (lactose). [b] Variance was less than 5% of the values presented. [c] $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{lactose}/(k_{cat}/K_m)_{cellobiose}]$, where T = 298 K and R = 8.314 kJ mol^{-1} K^{-1}. $\Delta(\Delta G)$ is a measure for the difference in binding energy between cellobiose and lactose.

Catalytic Properties of N732 Variants. Table 2 lists the k_{cat} and K_m values for rCDH and the N732 variants (N732H/Q/A/E/D) with cellobiose or lactose as the substrate and cytochrome *c* as the electron acceptor. The k_{cat} values of the variants for cellobiose oxidation were 5−4000-fold lower than for rCDH, and the order of decrease was His < Gln < Ala < Glu < Asp. All variants, except N732A, exhibited higher K_m values for cellobiose, which ranged from 2.5- to 15-fold higher than that for rCDH. The k_{cat} values for lactose oxidation with the N732 variants were similar to that for

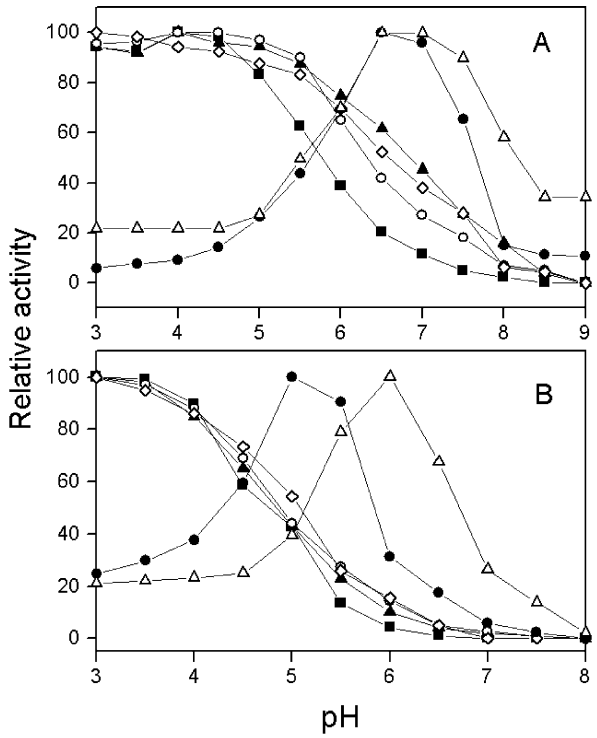

FIGURE 1: Dependence of cellobiose oxidation on pH by rCDH (■) and the N732 variants, N732H (▲), N732Q (○), N732A (◊), N732E (●), and N732D (Δ), with DCPIP (A) and cytochrome *c* (B) as electron acceptors. Reaction conditions are as described in the text. The highest level of activity observed for rCDH and the flavin domain variants over the range of pH values tested was set at 100%.

cellobiose oxidation (Table 2), as is observed for rCDH and the H689 variants. However, the K_m for lactose was affected in the N732 variants, increasing 5-fold for the N732A variant to 60-fold for N732E. The relative binding strength of the two substrates can be determined by calculating the difference in binding energy. Assuming that oxidation of lactose and cellobiose follows a similar reaction mechanism and the ratio of the configuration at the anomeric carbon (α/β) is similar for the two substrates, the difference in binding energy is similar to the change in activation energy ($\Delta(\Delta G)$). This change can be calculated using the relationship $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{lactose}/(k_{cat}/K_m)_{cellobiose}]$ (*30*). This shows that for the wild-type enzyme lactose binding was energetically less favorable than cellobiose binding by 7.2 kJ mol^{-1} and that a mutation of Asn732 weakened the binding of lactose by a further 2−4 kJ mol^{-1} (Table 2).

pH Dependence of Activity. Wild-type CDH exhibits an acidic pH optimum with a broad maximum between pH 3 and 5 for the oxidation of cellobiose with DCPIP (Figure 1A) (*9*, *25*). Activity gradually decreases above pH 5. Hence, the specific activity for rCDH and the flavin domain variants was measured at pH 4.5 (Table 3). All H689 variants (data not shown) exhibited a pH dependence similar to the wild-type enzyme. The mutations N732E and N732D introduce a carboxylate group, which may be ionized, and these variants exhibited a neutral pH optimum (6.5−7) with DCPIP as the electron acceptor (Figure 1A). In the presence of the one-electron acceptor cytochrome *c*, all flavin domain variants, except N732E and N732D, had a similar pH profile to that for rCDH, with the highest activity at pH 3, decreasing as the pH increased (Figure 1B). In contrast, the N732D and N732E variants exhibited optima at pH 6 and 5, respectively (Figure 1B).

Table 3: Specific Activity of rCDH and the H689 and N732 Variants at pH 4.5[a]

	Electron acceptor	
enzyme	cytochrome *c*	DCPIP
rCDH	9.1	17
N732H	1.4	3.7
N732Q	0.6	0.7
N732A	0.2	0.2
N732E	0.1	0.08
N732D	0.002	0.003
H689Q	0.002	0.007
H689N	0.002	0.007
H689A	0.002	0.007
H689V	0.001	0.003
H689E	0.005	0.01

[a] Assays were performed in 20 mM Na-succinate, pH 4.5, at room temperature, using 400 μM cellobiose and 12.5 μM cytochrome *c* or 35 μM DCPIP. Specific activity is calculated in μmol/min/mg.

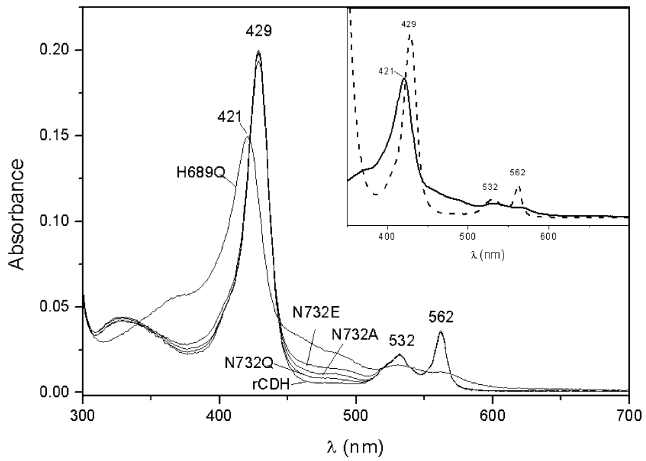

FIGURE 2: Electronic absorption spectra of cellobiose-reduced CDH holo-enzymes under aerobic conditions. The flavocytochromes rCDH and the CDH variants N732Q, N732A, N732E, and H689Q were treated with 500 μM cellobiose at pH 4.5 under aerobic conditions, and the resulting spectra were stable over at least 15 min. Insert shows the electronic absorption spectra of rCDH, oxidized (—) and dithionite-reduced (- - -).

Electronic Absorption Spectra of the H689 and N732 Variants. The UV−vis spectra of the oxidized and sodium-dithionite reduced H689 and N732 variants are similar to the wild-type protein rCDH (insert Figure 2) (*21*): the absorption bands at 421, 529, and 570 nm are attributed to a ferric, low spin heme, and the bands at 429, 532, and 562 are attributed to a ferrous, low spin heme (*27*). The weak absorbance between 450 and 500 nm indicates the presence of a flavin. Under aerobic and anaerobic conditions, cellobiose (500 μM) completely reduced the FAD and heme group in both rCDH and the N732H variant. In contrast, the addition of cellobiose did not reduce either the heme or the flavin in any of the H689 variants or the N732D variant, under aerobic conditions, and only the heme appeared to be slowly reduced under anaerobic conditions. Finally, under aerobic conditions the heme domain was completely reduced in the N732Q/A/E variants, whereas the flavin was only partially reduced (Figure 2). On the basis of the residual absorbance at 458 nm and an absorption coefficient of 10 mM^{-1} cm^{-1} (*10*), the FAD in N732Q, N732A, and N732E

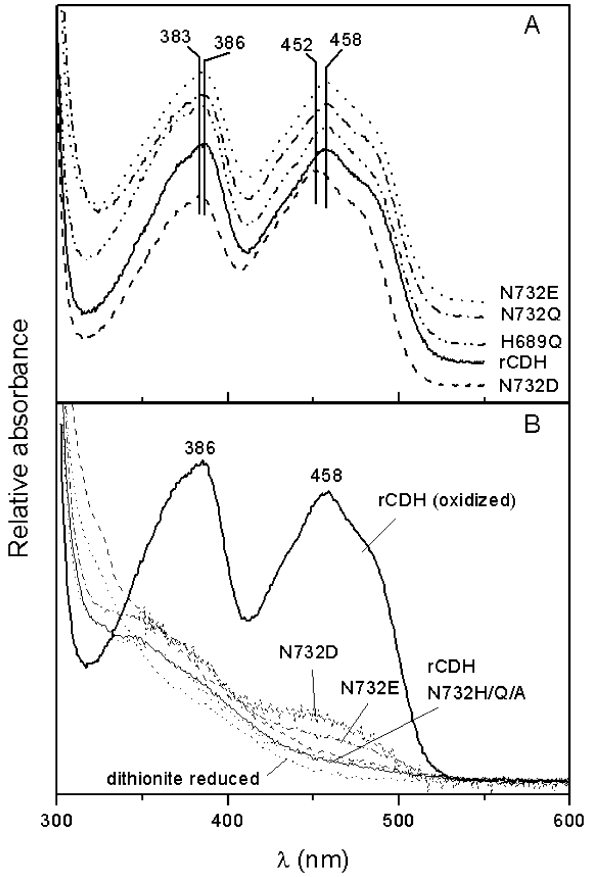

FIGURE 3: Electronic absorption spectra of the truncated flavin domain of rCDH and the variants N732H/Q/A/E/D and/or H689Q. (A) Oxidized; (B) reduced with cellobiose (30−300 μM) under anaerobic conditions. The relative absorbances are calculated using the absorbance at 458 nm for the flavin domain of rCDH as a reference. The spectra are offset in the *y* axis for clarity.

was reduced ~75, 50, and 35%, respectively. The addition of cellobiose under anaerobic conditions resulted in the complete reduction of both cofactors in these N732 variants.

Flavin Domain of rCDH and Variants. Because the heme absorbance dominates the UV−vis spectrum of a flavocytochrome such as CDH, the truncated flavin domains of rCDH and several flavin domain variants (N732H/Q/A/E/D and H689Q/A) were produced by limited proteolysis and anionic exchange chromatography. The molecular mass for the flavin domain of each variant, determined by SDS−PAGE, was approximately 60 kD, as was observed previously (*7*). The specific activities of each of the flavin domains for cellobiose oxidation in the presence of DCPIP were similar to those for the 90 kD enzymes, suggesting that the isolated flavin domains remained intact. The electronic absorption spectra of the flavin domain for most of the H689 and N732 variants were similar to the flavin domain of rCDH, with maxima at 386 and 458 nm (Figure 3A). The N732D variant exhibited blue-shifted maxima at 383 and 452 nm (Figure 3A). The addition of cellobiose to rCDH and the N732H/Q/A/E/D variants under anaerobic conditions resulted in bleaching of the absorbance, and it is estimated that >90% of the flavin was reduced (Figure 3B).

DISCUSSION

Flavoproteins, such as the flavin domain of cellobiose dehydrogenase (CDHdh), glucose oxidase (GOX), cholesterol oxidase (CHO), and monoamine oxidase, catalyze the oxidation of nonactivated alcohols and amines, which lack an electron-withdrawing group adjacent to the position of dehydrogenation. Two different mechanisms, a radical mechanism (*18*, *31*) and a hydride transfer mechanism (*18*, *32*), have been proposed to account for the dehydrogenation of these nonactivated substrates. In the radical mechanism, electrons are transferred from the substrate one at a time, whereas in the hydride transfer mechanism, two electrons are transferred in a single step. Despite this difference, both reactions appear to be assisted by the action of a general base (*18*).

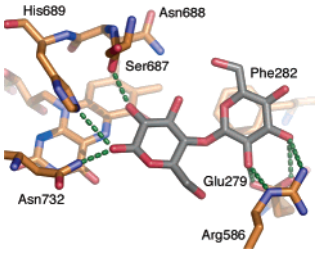

FIGURE 4: Active site of CDHdh complexed with cellobiose. The binding modes for the substrate were obtained by docking cellobiose in the active site of CDHdh (*13*). Atom colors: carbon, yellow (protein) and gray (cellobiose); oxygen, red; nitrogen, blue.

The tertiary structures of three members of the GMC oxidoreductase family, CDHdh (*13*), GOX (*17*, *33*), and CHO (*19*, *34*), reveal a highly conserved active site, including an active-site water and two semi-conserved residues, His and Asn (in GOX, the Asn residue is replaced by a His). In CDH, the active-site water, Wat1214, is hydrogen bonded to $N^{\epsilon 2}$-His689 and $N^{\delta 2}$-Asn732. This site is located near the isoalloxazine ring and at the end of a 12 Å channel that can accommodate a cellobiose molecule. Molecular modeling suggests that Wat1214 is expelled from the active site on binding of the substrate, and the anomeric β-hydroxyl CO1 of cellobiose is positioned between His689 and Asn732 (Figure 4). In this model, His689 acts as a general base, activating the substrate for oxidation by deprotonation of the β-hydroxyl. The anomeric α-hydrogen of cellobiose, pointing toward the FAD-N5, is optimally positioned for efficient hydrogen and electron transfer. The productive binding of the anomeric hydrogen is most likely assisted by the interaction between the β-hydroxyl and the two residues, His689 and Asn732. Previous mutant studies in GOX (*35*) and CHO (*36*−*38*) have demonstrated the importance of their active-site residues in catalysis. In this report, we have assessed the steric and electrostatic constraints of His689 and Asn732 of CDH from *P. chrysosporium* in binding and oxidation of the substrates cellobiose and lactose. The structural and kinetic properties of variants were assessed by kinetic and spectroscopic studies.

Role of His689. All the variants containing a substitution at His689 exhibit a significantly reduced oxidation rate for cellobiose (Tables 1 and 3), implicating this residue in catalysis. This is further supported by the apparent lack of any spectral changes in the heme and flavin cofactor in the presence of cellobiose (i.e., no apparent reduction of the flavin and heme cofactors were observed upon binding of cellobiose (Figure 2)). The low residual activity (>1000-fold decrease in k_{cat}) in all H689 variants, resulting from the absence of a base near the β-hydroxyl (Wat1204), suggests

that deprotonation facilitates oxidation. Thus, His 689 likely acts as a general base in the oxidation of cellobiose by CDH. This conclusion agrees with the site-directed mutagenesis studies on the catalytic histidine in CHO from *Streptomyces* sp. (*36*, *37*) and GOX from *Penicillium amagasakiense* (*35*), where a 100-fold decrease in catalytic activity for H447Q in CHO and a more than 10^5-fold decrease for Val and Ala substitutions in GOX were reported. The significant residual activity for the H447Q mutant was attributed to the presence of a base, Glu361, in the active site (*37*). No such residue is present in CDH.

Role of Asn732. The second catalytic residue, Asn732, would not act as a general base, but it appears optimally positioned to assist in the binding of the anomeric β-hydroxyl of cellobiose (Figure 4) (*13*). All Asn732 variants exhibit a reduced rate for cellobiose oxidation (Tables 2 and 3), implicating this residue in catalysis. The wide variation in the k_{cat} (0.004−2.7 s^{-1}) and the K_m (16−230 μM) for cellobiose among the N732 variants suggests that each substitution uniquely alters the substrate specificity of the enzyme (i.e., each substitution uniquely changes the active-site structure), affecting the binding as well as the positioning of the substrate with respect to the general base, His689, and the flavin, FAD-N5. Thus, the N732A variant exhibits a low K_m, indicating that it easily accommodates cellobiose. However, this variant lacks a hydrogen bond donor to assist in positioning the anomeric carbon. While the residues Gln and His may act as hydrogen bond donors, they are bulkier than Asn. The severalfold-lower k_{cat} and 2−3-fold higher K_m for N732Q and N732H suggest structural changes in the cellobiose−enzyme complex, affecting the binding and oxidation of the substrate. The Glu or Asp substitutions introduce possible hydrogen bond donors, or if ionized, a residue that may assist in the polarization of the β-hydroxyl of cellobiose. For example, in CHO, the residual activity in the H447Q variant is partly attributed to the presence of a second active-site base, Glu361, which is hydrogen bonded to the active-site water (*34*, *37*). Although the carboxylate residues in N732D and N732E appear ionized (see below), their presence apparently lends no advantage to CDH. Moreover, both substitutions result in a much higher K_m for cellobiose than is exhibited by the other variants, suggesting that the binding of substrate is significantly weakened in the N732E and N732D variants. Two possible explanations can be proposed. First, the presence of a negative charge may significantly alter the hydrogen bonding network within the active site, inducing steric and/or electrostatic hindrances for binding of the substrate. A second explanation is based on an extended hydrogen bonding network from Asn732 to Thr584 via Gln582. These residues are in close contact with Arg586, which forms two hydrogen bonds with the substrate in the enzyme−cellobiose complex (Figure 4). Disruption of this hydrogen bonding network, owing to a new negative charge, might alter the orientation of Arg586, thus weakening cellobiose binding. Overall, an Asn residue appears to be the optimal choice in the active site for CDH and in almost all other GMC oxidoreductases.

Lactose as Substrate. Lactose is an epimer of cellobiose, differing in configuration at the C4 of the nonreducing glucosyl unit (Glc2). In the cellobiose−enzyme complex, Glc2-C4 is located at the entrance of the substrate binding site (*13*). rCDH exhibits lower specificity toward lactose than cellobiose, which is attributed to the binding affinity for the substrates (i.e., the k_{cat} for oxidation is similar for both substrates, but the K_m is 15-fold higher for lactose (Tables 1 and 2)). The cellobiose−enzyme model (*13*) indicates the aromatic ring of Phe282 is stacked against the α face of Glc2 (Figure 4). For cellobiose, the hydroxyl at the Glc2-C4 position is oriented away from the aromatic ring of Phe282, whereas for lactose, this hydroxyl would be oriented toward the aromatic ring, hindering lactose binding. The kinetic data for the flavin domain variants with cellobiose suggest that Asn732 substitutions weaken the binding of the substrate, whereas the H689 variants exhibit a similar affinity for this substrate. The steady-state kinetic data for lactose oxidation with the flavin domain variants supports this. Furthermore, the K_m for lactose is greatly increased upon mutation of N732. Using the change in activation energy $\Delta(\Delta G)$ as a quantitative measure of the difference in binding energy between the substrates cellobiose and lactose (*30*), lactose binds 7.2 kJ mol^{-1} less favorably to the active site of rCDH than cellobiose (Table 2). This value is comparable to changes in free energy resulting from removal of a weak or strong hydrogen bond, shown to be 2−6 and 15−19 kJ mol^{-1}, respectively (*30*, *39*), and in CDH may be the result of the loss or weakening of hydrophobic, electrostatic, van der Waals, or hydrogen bonding interactions between the enzyme and the substrate. The additional 2−4 kJ mol^{-1} difference observed for the N732 variants suggests that an Asn residue may partially compensate for the weaker binding of lactose, whereas the N732 substitutions apparently do not.

pH Dependence. The results with the H689 and N732 variants (Figure 1) indicate that these residues do not control the pH profile for cellobiose oxidation. However, for an enzymatic reaction assisted by base catalysis, the protonation state of the base should be important. In CDH, His689 should be neutral in the catalytically active state. rCDH exhibits a pH optimum for cellobiose oxidation between 3 and 5 (Figure 1A), implying a greatly perturbed His pK_a from its value in aqueous solution (∼6). It is known that amino acid bases and carboxylic acids in proteins can exhibit anomalous pK_a, attributed to their microenvironments. For example, the general base His159 in the thiol protease papain exhibits an unusually low pK_a of ∼3.4 (*40*), attributed to a hydrophobic environment around the imidazole ring (*41*). In the case of CDH, His689 is indeed buried in a hydrophobic pocket of the active site, near the xylene moiety of the isoalloxazine ring (*13*). The results for N732D and N732E exemplify how the microenvironment around the general base, His689, can modulate its acid−base properties. The distance between Asn732-N^{δ} and His689-N^{ϵ} is ∼3.5 Å. The Asn732 carboxylate variants shift the pH optimum for cellobiose oxidation from pH 3−5 to 6−7 (Figure 1A). The introduced carboxylate groups may be ionized, creating a negative charge near His689. This would raise the pK_a of the His and thus shift the optimum to neutrality.

The shift in pH optimum upon introduction of a carboxylate group in the active site is of interest when comparing the catalytic properties of the CDHs from *P. chrysosporium* and *Humicola insolens* (*42*) since the *H. insolens* CDH exhibits a pH optimum closer to neutrality (*42*). While there is no crystal structure for the flavin domain from *H. insolens*, the primary sequence alignment of the five sequenced CDH genes (*2*, *14*, *43*−*46*) suggests that the catalytic site is

conserved in all CDHs, containing a His and an Asn. In addition, the N732E variant still exhibits an acidic pH optimum for cytochrome *c* reduction, whereas the alkaline CDH from *H. insolens* is most active at neutral/alkaline pH. Other differences in the active site may account for the different pH dependencies rather than an amide to acid substitution. The redox properties of the heme domains of the two CDHs are very similar (*28*), suggesting that differences within the flavin domain may control the interdomain electron transfer and the pH dependence.

Electronic Properties of the Flavin. The turnover rates for the two carboxylate variants, N732D and N732E, were somewhat surprising. Intuitively, the Glu substitution, which is electrostatically and sterically different from Asn, should have resulted in a weaker catalyst than the Asp mutation. In contrast, the N732E variant exhibits a higher k_{cat} (50-fold) than the N732D variant (Table 2). Under anaerobic conditions, the flavin cofactor is reduced by cellobiose in both variants (Figure 3B), but the rate of reduction is significantly lower in the N732D variant. The microenvironment around the isoalloxazine ring modulates various chemical properties of the flavin, including its electronic structure and reactivity (*47*). For example, flavin enzymes often have a positively charged entity in close contact with the N1−C2═O2 locus of the flavin (*48*). This regulates the redox properties of the cofactor, and it is proposed to stabilize the anionic form of the reduced flavin and thus to raise the redox potential (*32*). The pH profile (Figure 2) suggests that the carboxylate residues in the N732D and N732E variants are ionized, introducing a negative charge in the active site. This could significantly alter the electrostatic environment around the flavin, reducing the oxidizing power of the FAD cofactor. To probe the effect of these variants on the redox potential, the electronic absorption spectra of the truncated flavin domains of N732D and N732E were recorded and compared to rCDH and several of the other flavin domain variants (N732H/Q/A, H689Q/A) (Figure 3A). Previous mutant studies of flavin enzymes show that changes in the electronic absorption spectra are accompanied by changes in the redox properties of the flavin. For example, substitution of the catalytic Asn by Leu in CHO results in a drop of 76 mV in the redox potential, and the UV spectrum has a 4 nm red-shifted λ_{max} with respect to the wild type (*38*). In the old yellow enzyme, Thr34 forms a hydrogen bond with FMN-O4, and the T37A variant has a lower redox potential, supporting the role of Thr34 in stabilizing the reduced state (*49*). The shift in redox potential is accompanied by a shift in the absorption maxima to a shorter wavelength. Only the N732D variant exhibits a different flavin spectrum from rCDH, with absorption maxima blue-shifted to 383 and 452 nm (Figure 3A). Therefore, the Asp substitution may affect the redox properties of the flavin in CDH. The N732E (and other variants) exhibit a spectrum similar to that of rCDH, suggesting that their redox properties are not significantly altered.

Conclusions. Residues His689 and Asn732 are involved in catalysis by CDH from *P. chrysosporium*. The catalytic His appears to facilitate the oxidation of cellobiose and lactose and concomitant reduction of the flavin cofactor, functioning as a general base. The primary role of Asn732 appears to be to position the substrate 1α-H and 1β-OH relative to FAD and His689, respectively, which would promote deprotonation and oxidation. These conclusions are in agreement with those based on the crystal structure of the CDH flavoprotein in complex with a transition-state analogue (C. Divne, personal communication). The results also suggest that the proximity of a neutral residue, like Asn732, to the His689 and the isoalloxazine ring may control the acidity of the general base and the electrostatic environment around the flavin.

ACKNOWLEDGMENT

The authors thank C. Divne and B. M. Hallberg (KTH Royal Institute of Technology, Stockholm, Sweden) for help with the preparation of Figure 4.

REFERENCES

1. Henriksson, G., Johansson, G., and Pettersson, G. (2000) A critical review of cellobiose dehydrogenases, *J. Biotechnol. 78*, 93−113.
2. Li, B., Nagalla, S. R., and Renganathan, V. (1996) Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol. 62*, 1329−1335.
3. Dumonceaux, T., Bartholomew, K., Valeanu, L., Charles, T., and Archibald, F. (2001) Cellobiose dehydrogenase is essential for wood invasion and nonessential for kraft pulp delignification by *Trametes versicolor*, *Enzyme Microb. Technol. 29*, 478−489.
4. Ayers, A. R., Ayers, S. B., and Eriksson, K. E. (1978) Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*, *Eur. J. Biochem. 90*, 171−181.
5. Bao, W., Usha, S. N., and Renganathan, V. (1993) Purification and characterization of cellobiose dehydrogenase, a novel extracellular hemoflavoenzyme from the white-rot fungus *Phanerochaete chrysosporium*, *Arch. Biochem. Biophys. 300*, 705−713.
6. Habu, N., Samejima, M., Dean, J. F., and Eriksson, K. E. (1993) Release of the FAD domain from cellobiose oxidase by proteases from cellulolytic cultures of *Phanerochaete chrysosporium*, *FEBS Lett. 327*, 161−164.
7. Henriksson, G., Pettersson, G., Johansson, G., Ruiz, A., and Uzcategui, E. (1991) Cellobiose oxidase from *Phanerochaete chrysosporium* can be cleaved by papain into two domains, *Eur. J. Biochem. 196*, 101−106.
8. Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. (1991) Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families, *Microbiol. Rev. 55*, 303−315.
9. Samejima, M., and Eriksson, K. E. (1992) A comparison of the catalytic properties of cellobiose: quinone oxidoreductase and cellobiose oxidase from *Phanerochaete chrysosporium*, *Eur. J. Biochem. 207*, 103−107.
10. Rotsaert, F. A., Li, B., Renganathan, V., and Gold, M. H. (2001) Site-directed mutagenesis of the heme axial ligands in the hemoflavoenzyme cellobiose dehydrogenase, *Arch. Biochem. Biophys. 390*, 206−214.
11. Higham, C. W., Gordon-Smith, D., Dempsey, C. E., and Wood, P. M. (1994) Direct ^{1}H NMR evidence for conversion of β-D-cellobiose to cellobionolactone by cellobiose dehydrogenase from *Phanerochaete chrysosporium*, *FEBS Lett. 351*, 128−132.
12. Hallberg, B. M., Bergfors, T., Backbro, K., Pettersson, G., Herriksson, G., and Divne, C. (2000) A new scaffold for binding heam in the cytochrome domain of the extracellular flavocytochrome cellobiose dehydrogenase, *Structure 8*, 79−88.
13. Hallberg, B. M., Henriksson, G., Pettersson, G., and Divne, C. (2002) Crystal structure of the flavoprotein domain of the extracellular flavocytochrome cellobiose dehydrogenase, *J. Mol. Biol. 315*, 421−434.
14. Raices, M., Paifer, E., Cremata, J., Montesino, R., Stahlberg, J., Divne, C., Szabo Istvan, J., Henriksson, G., Johansson, G., and Pettersson, G. (1995) Cloning and characterization of a cDNA encoding a cellobiose dehydrogenase from the white rot fungus *Phanerochaete chrysosporium*, *FEBS Lett. 369*, 233−238.
15. Kiess, M., Hecht, H. J., and Kalisz, H. M. (1998) Glucose oxidase from *Penicillium amagasakiense*. Primary structure and comparison with other glucose-methanol-choline (GMC) oxidoreductases, *Eur. J. Biochem. 252*, 90−99.

16. Cavener, D. R. (1992) GMC oxidoreductases. A newly defined family of homologous proteins with diverse catalytic activities, *J. Mol. Biol. 223*, 811−814.
17. Wohlfahrt, G., Witt, S., Hendle, J., Schomburg, D., Kalisz, H. M., and Hecht, H. J. (1999) 1.8 and 1.9 Å resolution structures of the *Penicillium amagasakiense* and *Aspergillus niger* glucose oxidases as a basis for modelling substrate complexes, *Acta Crystallogr. D 55*, 969−977.
18. Li, J., Vrielink, A., Brick, P., and Blow, D. M. (1993) Crystal structure of cholesterol oxidase complexed with a steroid substrate: implications for flavin adenine dinucleotide dependent alcohol oxidases, *Biochemistry 32*, 11507−11515.
19. Vrielink, A., Lloyd, L. F., and Blow, D. M. (1991) Crystal structure of cholesterol oxidase from *Brevibacterium sterolicum* refined at 1.8 Å resolution, *J. Mol. Biol. 219*, 533−554.
20. Akileswaran, L., Alic, M., Clark, E. K., Hornick, J. L., and Gold, M. H. (1993) Isolation and transformation of uracil auxotrophs of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*, *Curr. Genet. 23*, 351−356.
21. Li, B., Rotsaert, F. A. J., Gold, M. H., and Renganathan, V. (2000) Homologous expression of recombinant cellobiose dehydrogenase in *Phanerochaete chrysosporium*, *Biochem. Biophys. Res. Commun. 270*, 141−146.
22. Sollewijn Gelpke, M. D., Mayfield-Gambill, M., Cereghino, G. P. L., and Gold, M. H. (1999) Homologous expression of recombinant lignin peroxidase in *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol. 65*, 1670−1674.
23. Alic, M., Mayfield, M. B., Akileswaran, L., and Gold, M. H. (1991) Homologous transformation of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*, *Curr. Genet. 19*, 491−494.
24. Alic, M., Kornegay, J. R., Pribnow, D., and Gold, M. H. (1989) Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol. 55*, 406−411.
25. Igarashi, K., Verhagen, M. F., Samejima, M., Schulein, M., Eriksson, K. E., and Nishino, T. (1999) Cellobiose dehydrogenase from the fungi *Phanerochaete chrysosporium* and *Humicola insolens*. A flavohemoprotein from *Humicola insolens* contains 6-hydroxy-FAD as the dominant active cofactor, *J. Biol. Chem. 274*, 3338−3344.
26. Morpeth, F. F. (1985) Some properties of cellobiose oxidase from the white-rot fungus *Sporotrichum pulverulentum*, *Biochem. J. 228*, 557−564.
27. Cohen, J. D., Bao, W., Renganathan, V., Subramaniam, S. S., and Loehr, T. M. (1997) Resonance Raman spectroscopic studies of cellobiose dehydrogenase from *Phanerochaete chrysosporium*, *Arch. Biochem. Biophys. 341*, 321−328.
28. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
29. Hazzard, J. T., Cusanovich, M. A., Tainer, J. A., Getzoff, E. D., and Tollin, G. (1986) Kinetic studies of reduction of a 1:1 cytochrome *c*−flavodoxin complex by free flavin semiquinones and rubredoxin, *Biochemistry 25*, 3318−3328.
30. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp 301−307, W. H. Freeman and Company, New York.
31. Silverman, R. B., Hoffman, S. J., and Catus, W. B., III (1980) A mechanism for mitochondrial monoamine oxidase catalyzed amine oxidation, *J. Am. Chem. Soc. 102*, 7126−7128.
32. Ghisla, S., and Massey, V. (1989) Mechanisms of flavoprotein-catalyzed reactions, *Eur. J. Biochem. 181*, 1−17.
33. Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., and Schomburg, D. (1993) Crystal structure of glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution, *J. Mol. Biol. 229*, 153−172.
34. Yue, Q. K., Kass, I. J., Sampson, N. S., and Vrielink, A. (1999) Crystal structure determination of cholesterol oxidase from *Streptomyces* and structural characterization of key active site mutants, *Biochemistry 38*, 4277−4286.
35. Witt, S., Wohlfahrt, G., Schomburg, D., Hecht, H. J., and Kalisz, H. M. (2000) Conserved arginine-516 of *Penicillium amagasakiense* glucose oxidase is essential for the efficient binding of β-D-glucose, *Biochem. J. 347*, 553−559.
36. Yamashita, M., Toyama, M., Ono, H., Fujii, I., Hirayama, N., and Murooka, Y. (1998) Separation of the two reactions, oxidation and isomerization, catalyzed by *Streptomyces* cholesterol oxidase, *Protein Eng. 11*, 1075−1081.
37. Kass, I. J., and Sampson, N. S. (1998) Evaluation of the role of His447 in the reaction catalyzed by cholesterol oxidase, *Biochemistry 37*, 17990−18000.
38. Yin, Y., Sampson, N. S., Vrielink, A., and Lario, P. I. (2001) The presence of a hydrogen bond between asparagine 485 and the π system of FAD modulates the redox potential in the reaction catalyzed by cholesterol oxidase, *Biochemistry 40*, 13779−13787.
39. Street, I. P., Armstrong, C. R., and Withers, S. G. (1986) Hydrogen bonding and specificity. Fluorodeoxy sugars as probes of hydrogen bonding in the glycogen phosphorylase-glucose complex, *Biochemistry 25*, 6021−6027.
40. Johnson, F. A., Lewis, S. D., and Shafer, J. A. (1981) Perturbations in the free energy and enthalpy of ionization of histidine-159 at the active site of papain as determined by fluorescence spectroscopy, *Biochemistry 20*, 52−58.
41. Kamphuis, I. G., Kalk, K. H., Swarte, M. B., and Drenth, J. (1984) Structure of papain refined at 1.65 Å resolution, *J. Mol. Biol. 179*, 233−256.
42. Schou, C., Christensen, M. H., and Schulein, M. (1998) Characterization of a cellobiose dehydrogenase from *Humicola insolens*, *Biochem. J. 330*, 565−571.
43. Xu, F., Golightly, E. J., Duke, K. R., Lassen, S. F., Knusen, B., Christensen, S., Brown, K. M., Brown, S. H., and Schulein, M. (2001) *Humicola insolens* cellobiose dehydrogenase: cloning, redox chemistry, and logic gate-like dual functionality, *Enzyme Microb. Technol. 28*, 744−753.
44. Moukha, S. M., Dumonceaux, T. J., Record, E., and Archibald, F. S. (1999) Cloning and analysis of *Pycnoporus cinnabarinus* cellobiose dehydrogenase, *Gene 234*, 23−33.
45. Dumonceaux, T. J., Bartholomew, K. A., Charles, T. C., Moukha, S. M., and Archibald, F. S. (1998) Cloning and sequencing of a gene encoding cellobiose dehydrogenase from *Trametes versicolor*, *Gene 210*, 211−219.
46. Subramaniam, S. S., Nagalla, S. R., and Renganathan, V. (1999) Cloning and characterization of a thermostable cellobiose dehydrogenase from *Sporotrichum thermophile*, *Arch. Biochem. Biophys. 365*, 223−230.
47. Yagi, K., Ohishi, N., Nishimoto, K., Choi, J. D., and Song, P. S. (1980) Effect of hydrogen bonding on electronic spectra and reactivity of flavins, *Biochemistry 19*, 1553−1557.
48. Fraaije, M. W., and Mattevi, A. (2000) Flavoenzymes: diverse catalysts with recurrent features, *Trends Biochem. Sci. 25*, 126−132.
49. Xu, D., Kohli, R. M., and Massey, V. (1999) The role of threonine 37 in flavin reactivity of the old yellow enzyme, *Proc. Natl. Acad. Sci. U.S.A. 96*, 3556−3561.

BI027092K