Mechanism of the Reductive Half-reaction in Cellobiose Dehydrogenase*

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The extracellular flavocytochrome cellobiose dehydrogenase (CDH; EC 1.1.99.18) participates in lignocellulose degradation by white-rot fungi with a proposed role in the early events of wood degradation. The complete hemoflavoenzyme consists of a catalytically active dehydrogenase fragment (DHcdh) connected to a b-type cytochrome domain via a linker peptide. In the reductive half-reaction, DHcdh catalyzes the oxidation of cellobiose to yield cellobiono-1,5-lactone. The active site of DHcdh is structurally similar to that of glucose oxidase and cholesterol oxidase, with a conserved histidine residue positioned at the re face of the flavin ring close to the N5 atom. The mechanisms of oxidation in glucose oxidase and cholesterol oxidase are still poorly understood, partly because of lack of experimental structure data or difficulties in interpreting existing data for enzyme-ligand complexes. Here we report the crystal structure of the Phanerochaete chrysosporium DHcdh with a bound inhibitor, cellobiono-1,5-lactam, at 1.8 Å resolution. The distance between the lactam C1 and the flavin N5 is only 2.9 Å, implying that in an approximately planar transition state, the maximum distance for the axial 1-hydrogen to travel for covalent addition to N5 is 0.8–0.9 Å. The lactam O1 interacts intimately with the side chains of His-689 and Asn-732. Our data lend substantial structural support to a reaction mechanism where His-689 acts as a general base by abstracting the O1 hydroxyl proton in concert with transfer of the C1 hydrogen to hydride to the re face of the flavin N5.

Cellobiose dehydrogenases (CDHs); EC 1.1.99.18) are extracellular fungal flavocytochromes that are believed to participate in lignocellulose degradation by fungi. They are oxidoreductases carrying protoheme and FAD cofactors bound to separate domains. In vivo, CDH from the white-rot Basidiomycete Phanerochaete chrysosporium depolymerizes cellulose, hemicelluloses, and lignin (Refs. 1–3; for review, see Ref. 4) as well as other polymers (5). The exact biological function of CDH has been a subject of lively debate, but recent results suggest that the enzyme is important for invasion and colonization of wood (6).

The catalytic site is located in the flavoprotein domain, where the reductive half-reaction proceeds by oxidation of β-cellobiose (apparent \( k_{cat} \) 15.7 s\(^{-1} \) and \( K_m \) 0.11 mM, see Ref. 7) to yield cellobiono-1,5-lactone (Fig. 1) and the concomitant two-electron reduction of FAD. In dilute aqueous solution, cellobionolactone hydrolyzes to cellobionic acid. Results from \(^1\)H NMR spectroscopy show that the product from cellobiose oxidation by CDH is unequivocally cellobionolactone, and thus, cellobionic acid is not formed on the enzyme (8). During the ensuing oxidative half-reaction, the flavin is re-oxidized by an electron acceptor, either directly or via the cytochrome domain (9). At present, the most favored mechanism for in vivo degradation of biopolymers by CDH is the reduction of ferric compounds present in wood in the presence of hydrogen peroxide to form hydroxyl radicals through a Fenton-type reaction (2, 10). The cytochrome domain has been implicated in this reaction (10) since the generation of hydroxyl radicals proceeds by one-electron reduction; however, the flavin domain is also able to generate hydroxyl radicals (1). Besides cellobiose, soluble cel-lulose, mannobiose and lactose, are good or acceptable substrates for CDH, whereas monosaccharides are poor substrates (7). CDH can use a large number of electron acceptors, however, reduction of oxygen is slow (11).

We recently reported the crystal structure of the dehydrogenase fragment of CDH without ligand (DHcdh) showing that it is closely related to that of members of the glucose-methanol-choline (GMC) family of oxidoreductases (12). The GMC oxidoreductases glucose oxidase (GOx (13, 14)) and cholesterol oxidase (ChOx (15, 16)) are similar in overall structure and active-site architecture to DHcdh (12). GOx catalyzes the oxidation of β-n-glucose to β-glucono-1,5-lactone. Thus, GOx and CDH perform similar chemistry in the reductive half-reaction. The bifunctional enzyme ChOx, however, catalyzes the oxidation and isomerization of cholesterol to 4-cholesten-3-one. Although CDH and GOx catalyze similar chemical reactions, the structural details of the catalytic site in the immediate vicinity of the flavin ring in CDH is more similar to those in ChOx; that is, two conserved residues (His and Asn) at the re face of the flavin ring in a similar conformation and with near identical geometry relative to the flavin N5 (12). In GOx, however, the
asparagine is replaced by a histidine residue. Based on modeling of cellobiose in the DH$_{cdh}$, active site, we suggested two glucosyl-binding sites at the $\alpha$ face of the isoalloxazine ring with the reducing end of cellobiose bound to the innermost site C close to the flavin ring and the non-reducing end of cellobiose residing in the distant site B (12).

Over the years, the most widely accepted reaction mechanisms for flavin-assisted dehydrogenation include the carbanion mechanism (17, 18), the radical mechanism (19, 20), and the hydride-transfer mechanism (21–23). In general, crystallographic data are not sufficient per se to conclusively discriminate between these mechanisms. However, with the increasing number of available ligand complexes for redox-active enzymes, the hydride-transfer mechanism is gaining in popularity as a general mechanism for dehydrogenation, although the radical mechanism has not been disproved. The only structure graphic data are not sufficient per se to conclude the hydride-transfer mechanism (21–23).

Overall Structure—The first crystal structure of a complex between the dehydrogenase fragment of CDH and Cblm, determined at 1.8-Å resolution, was reported elsewhere (7, 12). The authors propose a radical mechanism, although the radical mechanism has not been disproved. The only structure graphic data are not sufficient per se to conclude the hydride-transfer mechanism (21–23).

RESULTS

Electron Density and Refinement—Final refinement was done with REFMAC5 (29) at 1.8-Å resolution using anisotropic scaling, hydrogenals in their riding positions, and atomic displacement parameter refinement using the translation, libration, screw-rotation model. The flavin cofactor, inhibitor, glycosylation adducts, and the substrate and flavin binding domains of DH$_{cdh}$ (12) were defined as rigid bodies during translation, libration, screw-rotation refinement. The model contains 2 protein molecules (residues 215–755), two 6-hydroxylated FAD molecules; 5 N-acetylglucosamine residues (3 in molecule A and 2 in B); 2 Cblm molecules, and 1007 water molecules (533 for A, 473 for B, and 1 located on a non-crystallographic symmetry 2-fold axis). The bending angle of the isoalloxazine ring was calculated as described previously (12).

MODELING OF CELLOBIOSE—Cellobiose was modeled manually in the active site of DH$_{cdh}$ guided by the observed binding pattern for Cblm. To relieve geometric strain and impose favorable van der Waals contacts, the model was subjected to energy minimization with CNS (26) without the x-ray pseudo-energy term. During the energy minimization, only atoms within a sphere of 5.5 Å from the linking oxygen in the glycosidic bond were allowed to move. Atoms within a cushion of 3.5 Å around the sphere were refined with harmonic restraints.

EXPERIMENTAL PROCEDURES

Crystallization, Data Collection, and Refinement—DH$_{cdh}$ was purified and crystallized as described elsewhere (7, 12). Data were collected at 100 K from crystals soaked overnight in reservoir solution containing 1 M glycerol, 0.1 M HEPES, pH 7.0, 0.1 M magnesium acetate, and 0.1 M sodium citrate. Data collection and model refinement statistics are summarized in Table I. Data reduction and scaling were carried out using MOSFILM (24) and SCALAP (25), respectively. Our reported structure of DH$_{cdh}$ (Protein Data Bank code 1KDG (12)) was used as starting model for crystallographic refinement against DH$_{cdh}$-Cblm data. Initial refinement was done with CNS (26), and manual re-building was done with the program O (27). Starting coordinates for Cblm were generated using COOT (28) followed by manual fitting of the model to the electron density. Final refinement was done with REFMAC5 (29) at 1.8-Å resolution using anisotropic scaling, hydrogenals in their riding positions, and atomic displacement parameter refinement using the translation, libration, screw-rotation model. The flavin cofactor, inhibitor, glycosylation adducts, and the substrate and flavin binding domains of DH$_{cdh}$ (12) were defined as rigid bodies during translation, libration, screw-rotation refinement. The model contains 2 protein molecules (residues 215–755), two 6-hydroxylated FAD molecules; 5 N-acetylglucosamine residues (3 in molecule A and 2 in B); 2 Cblm molecules, and 1007 water molecules (533 for A, 473 for B, and 1 located on a non-crystallographic symmetry 2-fold axis). The bending angle of the isoalloxazine ring was calculated as described previously (12).

TABLE I

| Data collection | Resolution (Å) full range/outlier shell | Observations (measured/unique) |
|----------------|----------------------------------------|--------------------------------|
| 57.8–1.80/1.90/1.80 | 376,799,905/434 |
| Multiplicity | 4.0 (2.2) |
| Completeness (%) | 97.6 (65.4) |
| (L/|R|) | 6.7 (1.5) |
| R$_{free}$ (%) | 8.9 (49.8) |
| Refinement | Resolution range (Å) | 30–1.80 |
| Completeness for range (%) | 100.0 |
| R$_{work}$/number of reflections (work) | 0.146/83,130 |
| R$_{free}$/number of reflections (free) | 0.152/1894 |
| Number of non-hydrogen atoms | 9365 |
| Mean B values (Å$^2$) protein all atoms (A/B) | 8.8/8.9 |
| NCS r.m.s. deviation (Å) C=Vall protein atoms | 0.20/0.42 |
| NCS r.m.s. δB (Å$^2$) C=Vall protein atoms | 0.71/8 |
| r.m.s. deviation bond lengths (Å) angles (°) | 0.022/1.92 |
| Ramachandran plot outliers (%) | 1.8 |

* R$_{free}$ = $\Sigma_i$|Fo| - |Fclm| / $\Sigma_i$|Fo| 
* R$_{work}$ = $\Sigma_i$|Fo| - |Fclm| / $\Sigma_i$|Fo|

Overall Structure—The 1.5-Å crystal structure of DH$_{cdh}$ (Protein Data Bank code 1KDG) has been reported elsewhere (12). In brief, DH$_{cdh}$ consists of an FAD binding subdomain and a substrate binding subdomain (Fig. 2a). The entrance to the active site is located at the subdomain interface, where a 12-Å-long tunnel leads down to the flavin ring. The structures of DH$_{cdh}$ and DH$_{cdh}$-Cblm are nearly identical with root mean square deviation values (all atoms) of 0.35 and 0.34 Å for pair-wise least-squares comparisons of A and B molecules, respectively. The electron density for the protein as well as for the inhibitor is of excellent quality (Fig. 2b). The average residual B factor for the ligand (all atoms) is 8.2 and 8.4 Å$^2$ for the A and B molecule, respectively.

The Flavin Cofactor—As observed for DH$_{cdh}$, the non-covalently bound flavin cofactor in DH$_{cdh}$-Cblm is present as 6-hydroxylated FAD. The butterfly bending angle of the flavin ring is less pronounced in DH$_{cdh}$-Cblm (molecule A, 7°; molecule B, 11°) compared with DH$_{cdh}$ (22°). The flattening of the flavin ring appears to result from an induced fit where the N5 moves into the isoalloxazine plane to accommodate the incom-
Crudely oriented C1 atom of the lactam ring. The angle defined by the flavin N5 and N10 with the backbone nitrogen atom of Gly-310 is 136° and the N5-Gly-310 N distance is 3.2 Å.

Binding of Cellobionolactam—Inhibitor interactions are outlined in Fig. 3, a and b. The glucosyl-binding sites and the substrate-binding residues are located at the re face of the isoaaloxazine ring. The lactam moiety of Cblm, corresponding to the reducing end of cellobiose, is bound in site C with four protein residues and three solvent molecules within hydrogen-bonding distance of its exocyclic carboxyl and hydroxyl groups: O1-His-689 Ne², O1-Asn-732 Nδ², O2-Ser-687 O, O2-His-689 Ne², and O3-Asn-688 Nδ². The C3 and C6 hydroxyl groups can form one and two water-mediated hydrogen (H) bonds, respectively. Thus, a total of eight H-bonds are possible in site C. The endocyclic lactam nitrogen is positioned near the flavin N5 (3.2 Å) and O4 (2.9 Å). The C1 atom of the lactam moiety, which corresponds to the site of oxidative attack in cellobiose, binds in a position 2.9 Å in front of and below the N5-C4a locus of the isoaaloxazine ring, defining an angle of 108° (molecule A) and 110° (molecule B) with the N5-N10 atoms of the flavin ring. These values are in agreement with those typically observed in flavoenzymes (30). It should be noted that the lactam C1 and O1 are almost perfectly aligned with the flavin N5 and C4a, respectively (C1-N5, 2.9 Å; O1-C4a, 2.9 Å). The short distance (molecule A, 2.5 Å; molecule B, 2.6 Å) of the H-bond formed between the lactam O1 and His-689 Nε² suggests that this interaction is strong and that His-689 is suitably positioned to deprotonate the substrate hydroxyl group.

The glucosyl moiety of the lactid resides in site B where it forms a total of five ligand-protein H-bonds: O2-Glu-279 Oε², O2-Arg-586 Nε, O3-Glu-279 Oδ¹, O3-Arg-586 Nγ, and O6-Aasn-688 Nδ². The aromatic ring of Phe-282 has rotated (molecule A, 7°; molecule B, 11°) compared with the DHcdh structure to stack more planar with the B-site pyranose ring. Five solvent-mediated H-bonds are observed, one each for O2, O3, and O6 and two for O4. A total of 10 H-bonds and one planar hydrophobic stacking interaction contribute to binding in site B. Judged solely from the number of possible interactions, site B is likely to make substantial contribution to the binding of cellobiose, which is in line with reported kinetic constants for di- and monosaccharides (7).

Coupled Tyrosine-Serine Flipping—Only two residues undergo conformational change in response to inhibitor binding (Fig. 3b). In the non-ligated DHcdh structure, Tyr-609 is kept out of the C-site by H-bonds formed by its hydroxyl group with backbone atoms of Asn-732 and the Gln-582 side chain. The active-site tunnel is filled with solvent molecules, of which one water molecule binds in front of the flavin ring within H-bonding distance from His-689 Nε².

In the DHcdh-Cblm complex, however, the tyrosine side chain has discarded its H-bonding partners and moved into site C (Fig. 3b). In the new position, Tyr-609 resides below the lactam ring where Oη coordinates two ordered water molecules not present in the non-ligated structure. One water is located below the lactam O6, where it is involved in a H-bond network with Tyr-609 Oη, Thr-581 Oγ, Asn-732 Nδ², and the C6 hydroxyl group. The second water molecule is below the lactam C3 hydroxyl group and forms H-bonds to Tyr-609 Oη, the lactam O3, and another water molecule. The tyrosine and its two coordinated water molecules (colored yellow in Fig. 3, a and c) elevates the “floor” of site C.

The flipping of the tyrosine side chain imposes two additional changes in the active site as follows. (i) To accommodate the Tyr-609 side chain in its new position, the side chain of Ser-519 also flips and thereby exchanges its two H-bonding partners (Ser-519 Oα′-new water). The concomitant flips of Tyr-609 and Ser-519 give rise to a local backbone-Cα displacement of 0.6 Å and 0.8 Å at residue 609 and 519, respectively; (ii) the re-positioning of the Tyr-609 side chain together with the presence of the ligand effectively traps a water molecule (Wat1366) in a position 3 Å from the Ce⁻¹-Nδ³ edge of the His-689 imidazole ring (Fig. 3, a–c), i.e. the imidazole side opposite to that interacting with the C1 hydroxyl in cellobiose. This water molecule is not present in the non-ligated structure.

**DISCUSSION.**

*Comparison of Cellobionolactam with Modeled Cellobiose—* Structurally, cellobionolactam resembles the product cellobionolactone, with the only difference that the endocyclic O5 oxygen is replaced by an N-H function (Fig. 1). In both compounds C1 is coplanar with O1, C5, and O5 (or lactam nitrogen). We thus assume that the tetrahedral configuration of the anemic center of cellobiose is changing toward a planarized...
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**Fig. 3. Ligand interactions of the DH<sub>Cblm</sub> active site.** a, schematic representation showing the active-site interactions. Atom colors are black for carbon, blue for nitrogen, and red for oxygen. Covalent bonds are colored yellow (protein) or green (ligand), and H-bonds are drawn as green, dashed lines. The H-bond indicated between Wat1366 (colored violet) and His-689 Cε is hypothetical and requires a rotation of 180° about χε in the histidyl side chain. The water molecules that form the floor below the lactam moiety are drawn as yellow spheres. For clarity, H-bonds between ligand and water molecules other than those shown have been omitted as well as intramolecular H-bonds in the cellobionolactam molecule. The C1 atom in the ligand is labeled. The hydrophobic stacking interaction with Phe-282 is depicted with a red crest. The drawing was made with the program LIGPLOT (41). b, ligand interactions and the ligand-induced changes in the protein. The non-reducing end of the inhibitor is bound to site B (to the left), and the lactam ring in site C (right side). Interatomic distances that satisfy those of H-bonds (<3.2 Å) are depicted as dashed lines. For comparison, the DH<sub>Cblm</sub> structure without inhibitor (green) has been superimposed with DH<sub>Cblm</sub>, showing the ligand-induced changes in the protein discussed under “Results.” The water molecules coordinated by Tyr-609 and the H-bonds formed with the tyrosine are colored yellow. The water molecule (Wat1366) close to His-689 Cε is shown in violet color. A dashed line (violet) has been drawn to highlight the possible formation of a H-bond between Wat1366 and His-689 Ne2 given a rotation of 180° about χε. c, superposition of cellobionolactam (yellow) with modeled cellobiose (green). The 1-H and O1-H atoms (gray) are shown for the modeled cellobiose. For comparison, the position of 1-H in a TST has been drawn as a light-blue sphere connected to the lactam C1 by a dashed line. The water molecule close to His-689 Ne2 is shown as in b. The two tyrosyl-coordinated water molecules that form a floor below the lactam moiety of the ligand are shown as yellow spheres. For clarity, only selected interactions are shown in a-c. The drawings in b and c were made with the program Swiss-Pdb Viewer (42) and rendered with POV-Ray™ (www.povray.org).

Intermediate during its transition to a sp<sup>2</sup>-hybridized carbon in cellobionolactone.

Based on the Cblm binding pattern, we have produced a docking model of cellobiose in DH<sub>cdh</sub>. Minor steric restraints near the flavin N5 and His-689 Ne2 enable the planar lactam C1=O to penetrate somewhat farther (0.3–0.4 Å) into site C compared with the equatorial O1 group of cellobiose (Fig. 3c). The C1-N5 distance is 2.9 Å in the cellobiose model, and the shortest distance that the C1 hydrogen (1-H) would need to traverse to bind covalently to N5 is, thus, roughly 0.9–1.0 Å. In the Cblm complex, the corresponding transfer distance (imagine a 1-H as in the TST) would be slightly less, 0.8–0.9 Å. Although these distances do not differ significantly, the precise geometry of the 1-H relative to N5 is slightly different in cellobiose as compared with a TST in that 1-H in a TST would be closer to being aligned so as to interact with the lowest unoccupied molecular orbital of the flavin (Fig. 3c). The assumption that the observed inhibitor binding is also valid for a TST implies that upon approaching the TST the substrate slides slightly deeper into the active site. This results in a better alignment of the 1-H with the flavin lowest unoccupied molecular orbital. Similar to what has been suggested for several other flavoproteins (30), the resulting negatively charged flavin hydroquinone may be stabilized at the N1-C2–O locus of the isoalloxazine ring by the positive dipole of the C-terminal α helix. These small but distinct differences in binding may be interpreted as the oxidative site favoring a partially planar TST mimicked by the lactam ring in our structure.

Mechanistic Implications—Similar specific relative geometry of the substrate and cofactor-reactive groups has been observed in crystal structures of ligand complexes for nicotinamide-dependent (31), flavin-dependent (32), and quinone-dependent (33) oxidoreductases, all of which have been assigned a hydride-transfer mechanism. From a purely structural viewpoint, the close proximity between C1 and N5 together with the relative geometry of the atoms appears to favor a general base-catalyzed hydride-transfer mechanism (Scheme 1, panel a). General base-assisted deprotonation of the C1 hydroxyl group by His-689 in concert with the expulsion of 1-H as hydride via a planar or nearly planar TST would be entirely

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From the image provided, it seems to be a scientific document discussing the mechanism of cellobiose dehydrogenase, focusing on the interactions and binding of the ligands (cellobiose) in the active site of the enzyme. The text explains the structural and geometrical implications of the binding, particularly highlighting the differences between the cellobiose and the TST (transition state trajectory) models. It also mentions the role of the histidyl residue (His-689) in the deprotonation process and the alignment of 1-H with the flavin molecule during the transition to a more planar structure. The document provides a detailed schematic representation of these interactions, aiding in the comprehension of the mechanism.
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The carbamion mechanism in its classical implementation requires that His-689 abstracts 1-H as a proton, resulting in a substrate carbamion. The carbamion then performs a nucleophilic attack at N5 to form a covalent C1-N5 adduct. The subsequent elimination reaction proceeds by the concomitant abstraction of the O1-H as a proton by His-689, resulting in a flavin radical and a substrate radical. The subsequent step involves a transfer of the 1-H as a hydrogen radical to N5. Although the structure is compatible with the radical mechanism, the two radical species implied have not been demonstrated. The failure to detect the radical species spectroscopically does not, however, provide conclusive evidence against the radical mechanism. The formation of a cellbioisyl radical may be slow and its subsequent decomposition rapid, making detection of an electron spin resonance signal difficult. We may conclude that a physically meaningful difference between the hydride and the electron-transfer mechanisms hinges upon the temporal sequence, or concertedness, of events.

Studies on Structurally Unrelated Enzymes with Similar Substrate Specificity—The structure of soluble glucose dehydrogenase from Acinetobacter calcoaceticus in complex with its substrate, β-D-glucose, has been reported by Oubrie et al. (33). This enzyme catalyzes the oxidation of β-D-glucose to glucono-lactone but uses pyrroloquinoline quinone as cofactor. Similar to what is discussed here for CDH, a hydride-transfer mechanism was assigned to soluble glucose dehydrogenase (33) based on the specific orientation of 1-H relative to the C5 in pyrroloquinoline quinone (corresponding to the flavin N5 in CDH). In soluble glucose dehydrogenase, the distance between the substrate C1 and pyrroloquinoline quinone C5 is 3.2Å, implicating a transfer distance of 1.2Å for 1-H. The overall structure of CDH and soluble glucose dehydrogenase (Protein Data Bank code 1CQ1) and their active sites display no obvious similarity, but nevertheless, interesting details emerge when superimposing the active sites. A superposition with reference to C1 in the two enzyme complexes aligns the C5 of the pyrroloquinoline quinone cofactor in soluble glucose dehydrogenase within 0.7Å of the flavin N5 in CDH. The Ne² atoms of the proposed active base in soluble glucose dehydrogenase (His-144) and CDH (His-689) are only 0.4Å apart, and the distance between Asn-732 Nδ² in CDH and Arg-228 Nη² in soluble glucose dehydrogenase is 1.7Å. Thus, the precise geometry of the tetrad defined by the hydrogen acceptor of the cofactor, the C1, the proton acceptor of the general base catalyst, and the assisting residue coincide remarkably well despite different structure and cofactor dependence.

Studies on Structurally Related GMC Oxidoreductases—Although no results are available from site-directed mutagenesis studies on CDH, the residues proposed to participate in catalysis (12) have been mutated in the related enzymes GOx and ChOx. In B. sterolicum ChOx (35), Streptomyces ChOx (36), and Penicillium amagasakiani GOx (37), replacement of the proposed catalytic base (His-689 in CDH) resulted in enzyme variants with drastically reduced or abolished catalytic performance as measured by $k_{cat}$, whereas $K_m$ values were practically unaffected, thus supporting the assignment of His-689 in CDH as a general base catalyst. On the other hand, mutation of the Asn-732 counterpart in Streptomyces ChOx (N480A, N480Q) and P. amegasakiani GOx (H563A, H563V) resulted in inactive enzymes. In the light of the present structure and the mutant data for GOx and ChOx, we propose a dual role for Asn-732 where it (i) helps to position the substrate with respect to the flavin, and (ii) by offering a H-bond to O1, also facilitates proton abstraction by His-689.

The only crystal structure available for a GMC oxidoreductase-ligand complex is that of B. sterolicum ChOx with bound dehydroisandrosterone (16). In ChOx, His-447 (His-689 in CDH) has been proposed to activate a water molecule (Wat541) for nucleophilic attack on the substrate. This water molecule occupies the position of the substrate C1-O1 group in CDH. Thus, CDH and ChOx share the same reaction geometry, although the reaction in ChOx is suggested to be relayed through a water molecule. For GOx, no experimentally determined complex with substrate or substrate analogue is available, but β-D-glucose has been modeled in the active site (14, 37), resulting in a position of the substrate relatively similar to that of the C-site glucosyl moiety of Cblm in CDH; the substrate and the
catalytic residues are positioned at the re face of the flavin ring, and the C1 hydroxyl group is equidistantly positioned between His-689 and Asn-732 (His-520 and His-563 P. amagasakiense GOx). Molecular dynamics calculations of a glucose-GOx complex with a water-mediated interaction between the substrate O1 and the active histidine similar to that observed for ChOx resulted in expulsion of the water molecule, suggesting that a water- relayed mechanism is unlikely in GOx (14). Thus, direct interaction between the proposed catalytic base and the substrate is in agreement with our observed mode of Cbl binding to CDH.

**Induced Fit and Water Trapping**—Before another reductive half-reaction can occur, a total of two electrons acquired by the flavin needs to be transferred to an electron acceptor during the ensuing oxidative half-reaction, and the hydrogens transferred to N5 and His-689 Nε2 have to be suitably disposed of. For the N5 hydrogen, the most probable destination of a proton is to bulk water concomitantly with two single-electron or a two-electron transfer upon flavin re-oxidation (depending on the electron acceptor used). In the case of the proton withdrawn from the substrate 1-hydroxy group by His-689, a H3O+ ion triggers the Tyr-609 side chain to swing out from site below the ligand in site C. This imposes an effective restriction of this site to perfectly accommodate the lactam ring and thereby induce an optimal fit of the TST-like ligand to the protein. The movement of the Tyr-609 side chain into site C appears to occur in response to Cbl binding and results in the entrapment of a water molecule (Wat1366) close to His-689 Cε3 (Fig. 3, a–c). In the non-ligated structure, the position of Wat1366 is occupied by the CZ-OH group of Tyr-609. The water molecule within H-bonding distance to the imidazole group of the proposed catalytic base in the ligand structure, and it may, thus, serve as a secondary proton acceptor. The substrate proton acquired by His-689 can be transferred to Wat1366 by a 180° rotation about χ2 of the imidazole. In DHααCblm, Wat1366 interacts at the center of the aromatic ring of Tyr-609, which is likely to increase its affinity for the extra proton at His-689. A H2O−· r interaction (38) with the tyrosyl ring may, thus, promote proton transfer to Wat1366 and stabilize the resulting oxonium ion. The next step may be (i) that the oxonium ion triggers the Tyr-609 side chain to swing out from site C and resume its original position and thereby displace the product from the active site, and (ii) that product departure itself triggers the tyrosine side chain to leave the active site and release the oxonium ion to exchange a proton with bulk water, or (iii) that protonation of the histidine and the subsequent flip of the imidazole ring forces the product to leave due to an emerging unfavorable contact between the product carbonyl oxygen and the e-1 carbon of His-689 as a consequence of Ce4 assuming the position of Ne2.

Nevertheless, the tyrosine flip clearly helps to induce an optimal fit of the catalytic site for the inhibitor, and it generates a cavity for a solvent molecule that may accept a proton from the catalytic base. Wat1366 makes no direct contact with the site of oxidative attack, and hence, the C1-N5 pathway of a presumed hydride ion is completely shielded from water. It should be stressed that the position of Wat1366 in DHααCblm is completely different from that of the water molecule bound in front of His-689 in the non-ligated structure. This stresses the inherent difficulty in assigning catalytic roles to active-site water molecules in the absence of ligand or when non-authentic binding of ligand occurs. Thus, the water molecule implicated in catalysis by ChOx (16) should be carefully evaluated, although the dual function of ChOx may actually justify the presence of a catalytic water positioned between the substrate and the catalytic histidine.

**CONCLUSIONS**

The crystal structure of the CDH flavoprotein with bound inhibitor provides support for a hydride-transfer mechanism for dehydrogenation with His-689 acting as a general base catalyst, deprotonating the equatorial 1-hydroxyl group. The axial 1-H is aligned for a concerted hydride transfer from C1 to N5 via a transition state characterized by partial planarization of C1. The binding of the inhibitor is in agreement with hydrogen transfer at the re side of the flavin ring. We also suggest that Asn-732 is bifunctional in that it both participates in the formation of a productive enzyme-substrate complex and that it supports deprotonation by His-689 by serving as a H-bond donor to the 1-hydroxyl group. From a structural and biochemical viewpoint, the reaction mechanism is simple and requires only minor structural changes in the substrate and protein in order for the 1-H to be expelled. The 1-hydrogen would then need to traverse a distance of less than 1 Å for covalent attachment to the flavin N5 atom. Although the perfect setup of the active site for hydride transfer is evident, we cannot rule out the possibility of a radical mechanism using structural data alone. The results provide a structural platform for the use of conventional biochemical and biophysical techniques as well as quantum mechanical and classical molecular mechanics approaches to further investigate the molecular mechanism of cellulosioxidation by CDH.

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