Structural Basis for Substrate Binding and Regioselective Oxidation of Monosaccharides at C3 by Pyranase 2-Oxidase*

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Pyranose 2-oxidase (P2Ox) participates in fungal lignin degradation by producing the H2O2 needed for lignin-degrading peroxidases. The enzyme oxidizes cellulose- and hemicellulose-derived aldopyranoses at C2 preferentially, but also on C3, to the corresponding ketoadoses. To investigate the structural determinants of catalysis, covalent flavinylation, substrate binding, and regioselectivity, wild-type and mutant P2Ox enzymes were produced and characterized biochemically and structurally. Removal of the histidyl–FAD linkage resulted in a catalytically competent enzyme containing tightly, but noncovalently bound FAD. This mutant (H167A) is characterized by a 5-fold lower kcat and a 35-mV lower redox potential, although no significant structural changes were seen in its crystal structure. In previous structures of P2Ox, the substrate loop (residues 452–457) covering the active site has been either disordered or in a conformation incompatible with carbohydrate binding. We present here the crystal structure of H167A in complex with a slow substrate, 2-fluoro-2-deoxy-D-glucose. Based on the details of 2-fluoro-2-deoxy-D-glucose binding in position for oxidation at C3, we also outline a probable binding mode for D-glucose, D-xylose, and L-sorbose are good or reasonably good substrates, and D-galactose and L-arabinose perform poorly as substrates (7). Based on the catalytic efficiency, Kcat/Km for d-glucose (d-Glc) is the best substrate for T. multicolor P2Ox (7). Substrates that are oxidized at C3 were analyzed for P. gigantea P2Ox and include 2-deoxy-d-glucose, 2-keto-d-glucose, and methyl β-d-glosides (13, 10). That oxidation can take place either at C2 or at C3 presupposes two distinct, productive binding modes (referred to here as C2ox and C3ox) for a monosaccharide in the P2Ox active site.

Pyranose 2-oxidase (P2Ox),† pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10) is a flavin adenine dinucleotide (FAD)-dependent oxidase present in the hyphal periplasmic space (1) of wood-degrading basidiomycetes (2, 3). These fungi are the only known microorganisms that are capable of fully mineralizing lignin, and P2Ox has a proposed role in the oxidative events (4) of lignin degradation by providing the essential co-substrate, H2O2, for lignin and manganese peroxidases (5, 6). An alternative hypothesis assigns a role for P2Ox in both H2O2 production and in the reduction of quinones in the periplasm or in the extracellular environment (7). P2Ox from the white-rot fungi Trametes multicolor (Trametes ochracea) and Peniophora gigantea are hitherto the most studied biochemically (7–10) and structurally (11, 12).

P2Ox oxidizes a broad range of carbohydrate substrates that are natural constituents of hemicelluloses, allowing most ligno-cellulose-derived sugars to be utilized. Substrates can be oxidized regioselectively at the C2 position, although some oxidation at C3 can occur as a side reaction (10). For C2 oxidation, d-glucose, d-xylose, and l-sorbose are good or reasonably good substrates, and d-galactose and l-arabinose perform poorly as substrates (7). Based on the catalytic efficiency, Kcat/Km for D-glucose (D-Glc) is the best substrate for T. multicolor P2Ox (7). Substrates that are oxidized at C3 were analyzed for P. gigantea P2Ox and include 2-deoxy-d-glucose, 2-keto-d-glucose, and methyl β-d-glosides (13, 10). That oxidation can take place either at C2 or at C3 presupposes two distinct, productive binding modes (referred to here as C2ox and C3ox) for a monosaccharide in the P2Ox active site.

P2Ox from T. multicolor is homotetrameric with a molecular mass of 270 kDa (7) where each of the four subunits carries one FAD molecule bound covalently to N5 (i.e. N3) of His167 via its 8-α-methyl group (14, 11). The cofactor displays typical absorption maxima at 456, 345, and 275 nm (7). The reaction mechanism of P2Ox is of the type Ping Pong Bi Bi typically found in flavoprotein oxidoreductases (15, 16). In the reductive half-reaction, an aldopropyranose substrate reduces the FAD cofactor to

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* The atomic coordinates and structure factors (codes 2IGK, 2IGM, 2IGN, and 2IGO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: P2Ox, pyranose 2-oxidase; nsP2OxACT, non-steady-state P2Ox; CDH, cellobiose dehydrogenase; 2FG, 2-fluoro-2-deoxy-D-glucose; Mes, 4-morpholiniumethanesulfonic acid; ETF, electron transfer flavoprotein; ABTS, 2,2′-azinobis(3-ethylthiazoline-6-sulfonic acid).

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yield reduced flavin (FAD$_{\text{H}_2}$) and 2-ketoaldehyde (aldos-2-ulose) as a result of oxidation at position C2 of the sugar substrate (1, 10). The ensuing oxidative half-reaction involves the re-oxidation of FAD$_{\text{H}_2}$ by the second substrate, O$_2$, to yield H$_2$O$_2$ and the oxidized cofactor (Reactions 1 and 2).

$$\text{FAD} + \alpha\text{-glucose} \rightarrow \text{FADH}_2 + 2\text{-keto-}\alpha\text{-glucose}$$  
**REACTION 1**

$$\text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2$$  
**REACTION 2**

We have reported recently the 1.8-Å crystal structure of *T. multicolor* P2Ox in complex with acetate (nsP2Ox$_{\text{ACT}}$), a competitive inhibitor (11). The overall subunit structure of P2Ox belongs to the glucose-methanol-choline (GMC) family of FAD-dependent oxidoreductases (17, 18) with the cellobiose dehydrogenase (CDH) flavoprotein from *Phanerochaete chrysosporium* as the closest family relative (11). The GMC family is also represented by, among others, the enzymes glucose 1-oxidase from *Aspergillus niger* (19) and cholesterol oxidase from *Brevibacterium sterolicum* (20).

The active site of P2Ox was found to contain the conserved catalytic His-Asn center also present in CDH and cholesterol oxidase (His-Asn pair in glucose 1-oxidase). A loop that comprises residues 452DAFSYG457 (ab hinc, substrate loop) closes off the active site and interacts closely with the acetate ligand trapped in a pocket in front of the flavin cofactor (11). In *silico* modeling of substrate demonstrated that the observed loop conformation is not compatible with binding of a monosaccharide substrate, and that this restriction is mainly due to the position of the Phe$_{454}$ and Tyr$_{456}$ side chains. Thus, we predicted that the loop must be dynamic and allowed to undergo a major conformational change to bind a sugar (11), and that only when binding smaller, structurally compatible molecules such as acetate, or electron-acceptor substrates such as dioxygen, the loop would be in an ordered, closed conformation as that observed for nsP2Ox$_{\text{ACT}}$ (11). Moreover, the dynamic substrate loop appeared as a suitable candidate for discriminating between different carbohydrate substrates, for different orientations of the same carbohydrate, as well as for electron-donor substrate (carbohydrate) versus electron-acceptor substrates (dioxygen, quinone compounds, and others) (18).

Here we report the cloning, heterologous overexpression, biochemical characterization, and crystal-structure analyses of recombinant wild-type and mutant forms of *T. multicolor* P2Ox. The mutant variants discussed include the His-tagged asparagine replacement of the catalytic His$_{\text{548}}$ (His$_{\text{548}}$-H548N), the alanine replacement of His$_{\text{597}}$ responsible for covalent flavinylation (His$_{\text{597}}$-H167A), and the variants probing regioselective oxidation, *e.g.* alanine replacements of Asp$_{452}$ and Arg$_{472}$ as well as the combined double mutant.

The crystal structures of unliganded forms of the recombinant wild-type P2Ox (His$_{\text{597}}$-P2Ox), His$_{\text{548}}$-H548N, and His$_{\text{597}}$-H167A are described, as well as that of His$_{\text{597}}$-H167A in complex with the substrate analog 2-fluoro-2-deoxy-α-glucose (2FG). The results reported herein provide, for the first time, a detailed description of the structural determinants for substrate binding by P2Ox, and specifically, the role of the substrate loop in the C$_3$$_{\text{ox}}$ substrate-binding mode. Our results also offer an explanation for regioselective C2 versus C3 oxidation by P2Ox.

**EXPERIMENTAL PROCEDURES**

*Isolation of a Genomic Clone for T. multicolor P2Ox—* Genomic DNA from *T. multicolor* MB49 was isolated as described in a previous study (21). A fragment of the genomic p2o gene was amplified with PCR using primers Tmunp2oFOR2 (5’-gctgattaaacaaataaatcctagatct-3’) and Tmunp2oREV2 (5’-tgacctctctctccaagttcctccacca(aagtc-3’), derived from internal peptide sequences of purified P2Ox protein, genomic DNA as a template, and a standard PCR program (annealing temperature of 55 °C, 2-min extension). The resulting 1.2-kb fragment was cloned into plasmid pCR2.1 (TOPO TA Cloning Kit, Invitrogen) and verified as representing a fragment of the p2o gene by sequencing. A genomic library of *T. multicolor* was constructed in the IGEM11 bacteriophage vector (Promega, Madison, WI) according to the manufacturer’s instructions. The GOLD packaging extract (Stratagene, La Jolla, CA) and *Escherichia coli* LE392 host bacteria were used. The digoxigenin-labeled PCR-amplified fragments of the p2o gene were used to screen (PCR DIG probe synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany) the genomic library (21). Positive phage clones were analyzed by restriction digests and Southern hybridizations (21). DNA fragments covering the complete coding region of the p2o gene, including flanking 5’ and 3’ regions, were subcloned into pBluescript SK+ (Stratagene) and sequenced.

*Isolation and Expression of p2o cDNA—* *T. multicolor* MB49 was cultivated for 7 days in baffled Erlenmeyer flasks at 25 °C and 120 rpm in a medium containing whey powder (30 g/liter), peptone from casein (10 g/liter), and KH$_2$PO$_4$ (1 g/liter). Total RNA was isolated using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. 5 µg of total RNA was reverse-transcribed using the Reverse Transcription system (Promega) with an anchor primer (5’-ggc gcc cgc act act act att ttt ttt ttt tt-3’) for first strand synthesis. p2o cDNA was amplified with proofreading Pfu DNA polymerase, a Universal Primer specific for the multiple cloning site in the anchor primer, and a primer targeted to the sequence immediately upstream of the start codon of the p2o gene (Tmunp2ocDNAFOR, 5’-act aca cca gca tgt ct cc-3’; sequence information derived from genomic DNA). The resulting cDNA fragment of ~2 kb was cloned into pCR2.1 after addition of a 3’ overhanging adenine residue (Qiagen A Addition Kit) to give plasmid pCL12. For heterologous expression, the obtained cDNA was re-amplified using oligonucleotide primers Tmunp2oNCOR FOR (5’-act aca cca cca cta cta cca cta gct gac gc-3’), specific to the 5’-end of the coding region and containing an NcoI site, and Tmunp2oNotREV or Tmunp2oNotREV2 (5’-gag gcac gcg ggc cgc gac ctc-3’ and 5’-gag gcg ggc cgc tca ctg aat ctc-3’), for expression with and without the His$_{\text{597}}$ tag, respectively, specific to the 3’-end and containing a NotI site. The resulting amplon was digested with NcoI and NotI, gel-purified, and ligated with the appropriately cut and purified expression vector pET21 d(+) (Novagen, Merck Biosciences,
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Darmstadt, Germany) to give plasmid pH2. Verified expression vectors were subsequently transformed into the production strain BL21(DE3) (Novagen). Several clones were grown in 3 ml of M9 medium containing 10 g/liter glucose and 10 g/liter peptone from casein, induced by addition of 0.5% lactose (according to the pET-Vector system manual), and tested for presence of active P2Ox protein. Cultivation on a larger scale to provide material for biochemical characterization was done in 1-liter shake flasks containing 200 ml of medium at 200 rpm and 37 °C.

Construction of P2Ox Variants—Point mutations in plasmid pH2 were introduced using the GeneTailor Kit (Invitrogen). Mutation H167A was constructed with primers p2oGT-H167A-FOR (5’-tcggagccatgtctagcggcatgacctg-3’), carrying two base changes (bold characters, ca → gc), and p2oGT-H167A-REV (5’-agacagctcgcagacgcgcgtgaccgcctg-3’). The H548N mutation was constructed with primers p2oGT-H548N-FOR (5’-tccttcggccgg-3’) and p2oGT-H548N-REV (5’-acaagacaggctctcaaacctttgtgg-3’), and the D452A mutation was constructed with primers p2oGT-D452A-FOR (5’-acctcgacgccgcagctccgctcatcgtggactggg-3’) and p2oGT-D452A-REV (5’-ccgtgattctgagttgccacggctgtegttggctgg-3’), and the R472A mutation was constructed using primers p2oGT-R472A-FOR (5’-ctctagtcgacgccgcagctccgctcatcgtggactggg-3’) and p2oGT-R472A-REV (5’-ccagacgcaggcttgcttgg-3’). The mutant plasmids were isolated, and the mutations were confirmed by sequencing.

Enzyme Preparation and Purification—The pellet was re-suspended in a 3-fold volume of 100 mM phosphate buffer, pH 7.4, containing 1 mM NaCl, 10 mM imidazole, and 50 mM phenylmethylsulfonyl fluoride and passed through a French Press at 1,000 p.s.i. The resulting homogenate was centrifuged at 30,000 × g for 30 min at 4 °C to remove cell debris, and the supernatant was passed through a 50-ml Ni²⁺-immobilized metal ion affinity chromatography (IMAC) column (Chelating Sepharose Fast Flow, Amersham Biosciences). After washing the column with washing buffer, adsorbed proteins were eluted with imidazole buffer as per the manufacturer’s recommendations, and fractions were analyzed for P2Ox activity using the activity assay based on ABTS/peroxidase described in a previous study (7). In case of remaining impurities as assessed by SDS-PAGE using a PHAST-System (Amersham Biosciences), and if maximum homogeneity was desired, pooled fractions were passed through a 180-ml Superose 12 gel-filtration column (Amersham Biosciences).

Steady-state Kinetics and UV-visible Spectra—P2Ox activity was determined spectrophotometrically at 420 nm and 30 °C by measuring the formation of H₂O₂ for 3 min in a peroxidase-coupled assay using ABTS (ε₄₇₀ = 43,200 M⁻¹ cm⁻¹) as the chromogen (7). The standard assay mixture contained 1 mM ABTS in potassium phosphate buffer (50 mM, pH 6.5), 2 units/ml horseradish peroxidase, 100 mM D-Glc, and a suitable amount of the P2Ox sample, using air-saturated solutions. One unit of P2Ox activity is defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per minute under the given conditions. kinetic studies were carried out at 30 °C in phosphate buffer (pH 6.5) using the routine ABTS-peroxidase assay as outlined above. UV-visible spectra were recorded for P2Ox and H167A using a Beckman DU 800 spectrophotometer at room temperature. Measurements were performed in 50 mM phosphate buffer at pH 6.5 and 8.0. The enzyme was reduced using 10 mM D-Glc.

Reduction Potential Determination of rP2Ox and H167A—Reduction potential measurement of rP2Ox and the H167A mutant (both with and without expression vector derived polyhistidine tag) were carried out by using xanthine and xanthine oxidase as the reduction system (16). Briefly, a solution of enzyme, xanthine, standard dye, and xanthine oxidase (side arm) in a specially designed cuvette equipped with two side arms and a stopcock was made anaerobic by repeated cycles of evacuation and flushing with oxygen-free nitrogen. After anaerobiosis had been established, the reaction was initiated by adding xanthine oxidase from the side arm. For measurement of the wild type, indigo carmine (Eₒ = -116 mV) was used as standard dye, and a solution of dye and enzyme was reduced slowly over a period of 12 h. The reduction of His₆-rP2Ox was monitored at 459 nm, an isosbestic point for indigo carmine reduction, and indigo carmine reduction was monitored at 608 nm where the enzyme has no absorbance. These absorbance values were used to calculate the ratio of the reduced and oxidized species at various stages during the reduction process. The midpoint potential (Eₘₐₜ) can be calculated using the standard Nernst equation. For H167A, the same experiment was performed but with cresyl violet (Eₒ = -166 mV) as standard dye.

Reduction of His₆-rP2Ox and His₆-H167A by 2FG—The capability of 2FG to reduce the FAD cofactor in P2Ox was investigated. A solution of the enzyme (20 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C was made anaerobic as described above. Then, 2FG (147 μM) in the side arm was tipped into the enzyme solution. The reduction of His₆-rP2Ox was monitored at 456 nm, and kₘₐₜ was analyzed from a single-exponential decay of a plot of absorbance at 456 nm with time. The reduction of His₆-H167A by 2FG was measured by monitoring absorbance at 453 nm over a period of 4 days. All reduction reactions were kept in the dark to avoid light-induced reduction of the flavin.

Crystallization, Data Collection, and Refinement—Crystals of the recombinant proteins were obtained in the presence of 12–13% monomethylether polyethylene glycol 2000, 0.1 M Mes (pH 5.2), 50 mM MgCl₂, and 25% glycerol using the hanging-drop vapor diffusion method and microseeding. Equal volumes of protein solution (4 mg/ml in 20 mM Mes, pH 5.2) and reservoir were mixed to prepare the drops. For the ligand complex, 2FG was added by co-crystallization (H167A / 2FG, 12 mM 2FG). Data were collected at 100 K using synchrotron radiation.

Crystallographic refinement was performed with REFMAC5 (22) and included anisotropic scaling, calculated hydrogen scattering from riding hydrogens, and atomic displacement parameter refinement using the translation, libration, screw-rotation model. Corrections of the models were done manually with the guidance of σFo-2Fo and Fo-Fc electron-density maps. The same set of Rfree reflections was used throughout all refinement, and NCS restraints/constraints were not used. Model building, coordinate manipulation, and least-squares comparisons were made with the programs O (23) and Coot.
RESULTS

Isolation and Characterization of Genomic and cDNA Clones from T. multicolor—PCR amplification of T. multicolor genomic DNA using primers Tmp2ofor2 and Tmp2orev2, derived from sequence tags from the mature purified protein, yielded a 1.2-kb fragment, which was clearly identified as representing a part of the p2o gene by identification of several other sequence tags in the various reading frames. Screening of a genomic library of T. multicolor using this fragment as a probe yielded two positive clones, which appeared to contain identical chromosomal inserts, as was judged by restriction enzyme mapping. One of the two clones was further characterized, 4460 bp were sequenced and found to contain the complete coding sequence, including flanking 5′ and 3′ regions. Based on preliminary translation, which was aided by comparison to the Coriolus versicolor PROD sequence (GenBank™ accession number D73369) and by known sequence tags, the putative start codon was identified, and a cDNA fragment was amplified by reverse transcription-PCR based on the sequence information from the genomic DNA. The sequence of the fragment was found to be identical to a T. ochracea p2o sequence (GenBank™ accession number AY291124 (25)) that was deposited in GenBank™ during the course of this work. The genomic DNA (GenBank™ accession number AY753305) contains a coding region of 2723 bp, which is interrupted by 15 introns. The intron length varies between 51 and 62 bp, with an average length of 56, 5′- and 3′-splice sites generally conform to the established consensus sequences for fungal introns (26) GTR and YAG, with only three exceptions altogether (5′-GCA and 5′-GTC in introns VI and VIII, respectively, and AAG-3′ in intron XII). However, a high occurrence of the motif CTRA rather than the consensus CTRA (in 9 introns) in the putative lariat sequences can be observed. Exon V is unusually short (only 9 bp), as well as exon XI (20 bp). The only other available genomic sequence of a pyranose oxidase gene is from Phanerochaete chrysosporium, the entire genome of which was sequenced by the U.S. Dept. of Energy (Joint Genome Initiative, www.jgi.doe.gov). The P. chrysosporium gene contains 14 introns of very similar lengths, whose positions within the gene, however, are not quite identical to the respective positions in the T. multicolor gene. It also features a very short exon V (10 bp) in the same position as the Trametes gene. An investigation of the 5′-regulatory region of p2o using the programs PATCH and MATCH (hosted at the Expasy-MB-Server, www.expasy.ch) yielded several motifs similar to established transcription factor binding sites, notably two StuA-binding sites (−515 and −732) and two AbaA sites (−689 and −1025 (27)).

Heterologous Expression of p2o cDNA and Production of Recombinant Enzymes—We chose the pET expression system (24). Figs. 3 and 4 were prepared using PyMOL version 0.93 (pymol.sourceforge.net).

|          | k_{cat} | K_{m} | k_{cat}/K_{m} |
|----------|---------|------|--------------|
| nsP2Ox   | 45.4    | 0.72 ± 0.03 | 63.1 |
| His6-rP2Ox | 50.5    | 0.82 ± 0.03 | 61.6 |
| His4-H167A | 8.83    | 3.60 ± 0.58 | 2.45 |
| His6-H548N | 0.00109 | 1.10 ± 0.05 | 0.00099 |

*P2Ox purified from the natural source T. multicolor.

The mutant enzymes contain the His6 tag. Prior to kinetic measurements, the tagged proteins were treated with 100 mM EDTA at 4 °C overnight to eliminate the inhibitory effect of Ni^{2+}.

Steady-state Kinetics—To evaluate the effect of the mutations, steady-state kinetics of the mutants were investigated and compared with those of the wild type. With D-Glc as the substrate, the k_{cat} value of His6-rP2Ox purified by nickel-affinity chromatography was reduced by 25–50% compared with that of nsP2Ox, whereas only minor differences were observed for the K_{m} values (data not shown). The His_{6} tag is located at the C terminus of each of the four monomers, all of which are solvent-exposed on the surface of the tetramer, and thus, well separated from the internal cavity from which the four active sites are accessible. Thus, the tag itself is unable to interfere with catalysis through direct steric hindrance in or near the active site. After extensive treatment with EDTA of the His_{6}-tagged enzyme, the catalytic activity was essentially restored to that of nsP2Ox (Table 1). Thus, the inhibitory effect on catalysis must arise from Ni^{2+} originating from the immobilized metal ion affinity chromatography resin used in purification.

The His_{6}-H167A mutant displays a k_{cat} value corresponding to 17% of that of the wild type (His_{6}-rP2Ox) when measured using saturating concentrations of D-Glc (≥50 mM) as substrate. Binding of substrate is affected to some degree in His_{6}-H167A as shown by a 5-fold increase in K_{m} value. In contrast to the H167A mutant, the Asn replacement of His_{548} produces an essentially inactive enzyme with a 46,000-fold lower k_{cat} and 62,000-fold lower catalytic efficiency (k_{cat}/K_{m}) but with a K_{m} value that is only slightly affected compared with His_{6}-rP2Ox.
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UV-visible Spectra—UV-visible absorbance spectra of recombinant wild-type P2Ox displayed typical flavin maxima at 360 and 457 nm corresponding to the oxidized state. There was no significant difference at pH 6.5 and 8.0, or between His6-rP2Ox and nsP2Ox. As expected for the H167A mutant where the covalent linkage between the flavin and His167 has been eliminated, different absorption peaks, 385 and 450 nm, are observed compared with the wild type. Near identical values were obtained at pH 6.5 and 8.0, 25 °C.

Reduction Potential Determination of rP2Ox, His6-rP2Ox, H167A, and His6-H167A—The reduction potential of rP2Ox, His6-rP2Ox, H167A, and His6-H167A were measured to assess the effects of the affinity tag on activity, as well as of the His167 Nε-FAD C8M covalent bond on the redox property of P2Ox. Fig. 1A represents spectra obtained during anaerobic reduction of the His6-rP2Ox and indigo carmine using the xanthine/xanthine oxidase reduction system. The results suggest that the enzyme was reduced via a two-electron reduction process, because there is no sign of flavin semiquinone formation typically found in the long-wavelength or 370 nm region. Reduction of the enzyme was monitored by the decrease in absorbance at 459 nm, and the reduction of dye was monitored by decrease in absorbance at 608 nm. A plot of log(Ered/Eox) versus log(Dred/Dox) yielded a slope of 1.2 (inset to Fig. 1A), again indicating that the reduction process mainly occurred via a two-electron reduction. The midpoint potential calculated from this plot was −0.115 V (inset to Fig. 1A).

Fig. 1B shows the spectra obtained during anaerobic reduction of the His6-H167A mutant and cresyl violet using the xanthine/xanthine oxidase reduction system. Reduction of the enzyme was monitored at 389 nm, an isosbestic point of dye reduction, and reduction of the dye was monitored at 571 nm where the enzyme has no absorption. A plot of log(Ered/Eox) versus log(Dred/Dox) yielded a slope of 0.92 (inset to Fig. 1B), indicating that the reduction process occurred via a two-electron reduction. The midpoint potential calculated from this plot was −0.147 V (inset to Fig. 1B). Table 2 summarizes the redox potential values of rP2Ox, His6-rP2Ox, H167A, and His6-H167A, and shows that the E°m value of H167A is 32–45 mV lower than that of the wild type. Only minor differences were observed in E°m values for His6-tagged and non-tagged enzymes, which is in agreement with the tag being located on the surface, well separated from the active site.

Reduction of His6-rP2Ox and His6-H167A by 2FG—To assess whether 2FG is an inhibitor or a slow substrate, reduction experiments were performed using recombinant rP2Ox and H167A. Data in Fig. 2 show that reduction of His6-rP2Ox (Fig. 2A) occurs much faster than of His6-H167A (Fig. 2B), as judged by the kobs values of 0.0064 min−1 and 0.000027 min−1, respectively. Thus, 2FG can reduce both His6-rP2Ox and His6-H167A, indicating that 2FG is indeed a slow substrate for both enzymes. This also implies that the enzyme should be able to oxidize α-D-Glc at the C3 position and that the structure of His6-H167A2FG reported herein represents an authentic binding mode.

Crystallization, Data Collection, and Refinement—To circumvent the problem with tightly bound acetate in the P2Ox active site and allow for binding of carbohydrate ligands, the crystallization conditions reported previously (11, 18) were modified to exclude acetate in the buffer and mother liquor. In addition, due to the high sensitivity of the former P2Ox crystals

![FIGURE 1. Determination of reduction potentials. A, His6-rP2Ox. Line 1 is a spectrum of the enzyme and dye in oxidized form, whereas lines 2–7 are spectra during the reduction process. A spectrum of the completely reduced enzyme and dye was shown in line 8, Inset to A, the reduction of indigo carmine was monitored by a decrease in absorbance at 608 nm, and the reduction of His6-rP2Ox was monitored by a decrease in absorbance at 459 nm, allowing the ratio of reduced and oxidized species to be calculated. The reduction potential of His6-rP2Ox was calculated to be −115 ± 2 mV. B, His6-H167A mutant. Line 1 is a spectrum of the oxidized enzyme and dye, whereas lines 2–6 are spectra during the reduction process. A spectrum of the completely reduced enzyme and dye was shown in line 7. Inset to B, the reduction of cresyl violet dye was monitored by decrease in absorbance at 584 nm, and the reduction of H167A mutant was monitored by decrease in absorbance at 414 nm (an isosbestic point of dye), allowing the ratio of reduced and oxidized species to be calculated. A slope of the plot of log(Ered/Eox) versus log(Dred/Dox) was close to 1.0, and the reduction potential of H167A mutant was calculated to be −147 ± 2 mV.]

| Protein        | E°m (mV) |
|----------------|----------|
| rP2Ox          | −105 ± 1 |
| His6-rP2Ox     | −115 ± 3 |
| H167A          | −150 ± 2 |
| His6-H167A     | −147 ± 2 |
to treatment with cryo-protective agent, further optimization was performed to obtain crystals in the absence of acetate and presence of cryo-protectant. The recombinant enzymes (His6-rP2Ox, His6-H167A, and His6-H548N), crystallized in the presence of cryo-protectant, produced crystals in the same space group (P2₁) but with a unit cell of twice the size compared with that reported previously for T. multicolor P2Ox (18). Data collection statistics are summarized in Table 3. The C-terminal His₆ tag alone may be sufficient to alter the crystal packing to produce the larger unit cell; however, the addition of cryo-protectant to the mother liquor is also likely to be important.

Initial phasing of the new data sets was achieved by molecular replacement with the program AMoRe (28) using the T. multicolor P2Ox model (Ref. 11, PDB code 1TT0) as search probe. For the recombinant data sets in P2₁, a solution was obtained that included eight monomers per asymmetric unit (two functional homotetramers). All P2Ox subunits contain 8 histidyl-linked FAD molecules (with the exception of H167A), and in the case of the His₆-H167A₂FG complex, one 2FG molecule has been added per monomer. Refinement and model statistics are given in Table 3.

**Structure of Recombinant Proteins and Rearrangement of the Dynamic Substrate Loop**—The overall subunit and tetramer structure is very similar in recombinant and naturally occurring P2Ox. Except for the mutated residues, the overall structures, as well as the active sites, of His₆-rP2Ox, His₆-H548N, His₆-H167A, and His₆-H167A₂FG are identical within the coordinate errors expected. The only major difference for the models is seen in the substrate loop (residues 452–457). In nsP2OxACT (11), the substrate loop is well defined and closed, and all access to the active site is effectively prevented. In the His₆-H167A₂FG complex, however, the loop is again well ordered but fully open. In the unliganded states of His₆-rP2Ox, His₆-H167A, and His₆-H548N, the loop is open but with varying degrees of disorder. During the transition from the closed loop conformation in nsP2OxACT (Fig. 3A) to the fully open conformation in His₆-H167A₂FG (Fig. 3B), the loop undergoes a complete rearrangement.

**Binding of 2FG to H167A and Proposed Binding of D-Glc in the C2ox and C3ox Modes**—The conversion of D-Glc to 2-keto-D-Glc by T. multicolor P2Ox appears to proceed with 100% yield, indicating that no oxidation takes place at C3, and that the C3ox mode is discriminated against effectively. The only structural difference between the substrate analog 2FG and the natural substrate, D-Glc, is the substitution of the C2 hydroxyl group by a fluorine atom. In His₆-H167A₂FG, the electron density for the ligand is well ordered and unambiguous (Fig. 4A). The 2FG molecule is, however, oriented with the 2-fluorine atom (equivalent to the oxidizable C2 hydroxyl group in D-Glc), in a position where O3 is expected, and with the C3 hydroxyl group positioned where O₂ should bind to produce 2-keto-D-Glc with D-Glc as electron-donor substrate. Protein residues that participate in hydrogen bonds with the sugar are: His₅₄₈, Asn₅₉₃, Gln₄₄₈, and Val₅₂₆ (Fig. 3B and Table 4).

**TABLE 3**

Statistics for data collection and crystallographic refinement

|                     | His₆-rP2Ox | His₆-H548N | His₆-H167A | His₆-H167A₂FG |
|---------------------|------------|------------|------------|---------------|
| **Data collection** |            |            |            |               |
| Wavelength (Å)      | 1.095      | 0.939      | 0.968      | 1.092         |
| Beamline/temperature (K) | MAX-lab I711/100 | ESRF ID14-4/100 | MAX-lab I911-5/100 | MAX-lab I711/100 |
| α, β, c (Å)         | 168.8, 103.9, 169.3 | 169.4, 103.1, 168.9 | 168.1, 103.0, 168.6 | 168.6, 103.1, 169.0 |
| β (%)               | 106.36     | 106.08     | 106.42     | 106.30        |
| Resolution range (Å) | 39.2-1.70 (1.75-1.70) | 20.1-1.90 (2.00-1.90) | 39.0-1.65 (1.70-1.65) | 38.9-1.95 (2.00-1.95) |
| Unique reflections  | 608,237 (45,425) | 414,274 (59,831) | 659,653 (56,354) | 403,281 (29,439) |
| Multiplicity        | 4.6 (3.3)  | 3.7 (3.7)  | 4.6 (3.2)  | 4.4 (3.8)     |
| Completeness (%)    | 99.0 (89.1) | 97.0 (96.5) | 99.9 (99.9) | 99.8 (99.9)   |
| R/i                | 7.6 (68.8) | 7.4 (33.7) | 10.6 (52.1) | 7.1 (31.0)    |
| R_index (%)         | 40.1-1.80  | 20.1-1.90  | 39.1-1.65  | 40.1-1.95     |
| Completeness (%)    | 99.9       | 96.8       | 99.9       | 99.9          |
| R/i_restricted       | 17.7/512,167 | 15.2/417,976 | 17.2/653,089 | 18.4/399,232 |
| R/i_restricted (free) | 21.4/5,182 | 18.9/4,236 | 20.5/6,563 | 22.1/4,042    |
| Non-hydrogen atoms  | 39,852     | 41,263     | 41,766     | 38,613        |
| Mean B (Å²) al/protein | 23.1/22.8 | 19.6/18.4 | 23.7/22.4 | 27.8/27.8     |
| Root mean square deviation bond lengths (Å) | 0.020, 1.8 | 0.018/1.7 | 0.018/1.8 | 0.023, 1.9 |
| Ramachandran: favored/outliers (%) | 97.4/0.07 | 97.6/0.04 | 97.6/0.0 | 97.6/0.0    |

*a R_index = [Σw_r ||Fo-Fc||/Σw_r ||Fo||] × 100%.

*b R/i = [Σw_r ||Fo|| - ||Fc||]/Σw_r ||Fo||.

*c As determined by MolProbity (44).
The C2<sub>ox</sub> and C3<sub>ox</sub> modes are related by a 180° rotation about an axis intersecting midway between C2–C3 and C5–O5. Thus, for D-Glc, the following transformations produce structurally equivalent interactions: O1 → O4, O2 → O3, O4 → O1, and O5 → C5. However, because O5 does not make any interactions with the protein, the O5 → C5 transformation should not affect ligand binding to any appreciable extent, at least not from a purely structural point of view. Protein-ligand interactions that satisfy hydrogen-bonding distances are outlined for the observed C3<sub>ox</sub> orientation and the expected C2<sub>ox</sub> orientation (Fig. 4, B and C, and Table 4). If transforming the 2FG molecule as instructed above, a glucosyl molecule with essentially identical protein interactions is obtained. The most obvious difference is the position of the exocyclic O6 group, which forms no hydrogen bonds to protein atoms in His<sub>6</sub>-H167A<sub>2FG</sub>. In the rotated C2<sub>ox</sub> orientation, however, free rotation about the C6–O6 bond would easily bring the C6 hydroxyl group within hydrogen-bonding distance of Asp<sub>452</sub> O<sup>7</sup> and Arg<sub>472</sub> N<sup>ε</sup>. The distance from the site of oxidative attack in 2FG (C3 in His<sub>6</sub>-H167A<sub>2FG</sub>, equivalent to C2 in C2<sub>ox</sub> mode) to the FAD N5 atom...
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from the superimposition of His6-H167A2FG and nsP2OxACT, Waals interactions, with the bound acetate molecule. Judgingation in the active site to interact closely, through van der

tively, for these three substrates). The double mutation in His6-

is due to the rearrangement of the substrate loop where Asp452

tively) than when Arg472 was exchanged (His6-R472A: K_m

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appears to be the possibility of one or two additional protein

tact. The Thr169 O

— The rationale for product outcome for substrates oxidized at

Crystal Structure Complex of P2Ox with 2FG

is only 3.1 Å, supporting hydride transfer from C3 to N5 at the

To further corroborate the proposed interactions between the C6 hydroxyl group and the side chains of Asp452 and Arg472, the mutants D452A and R472A as well as the double mutant D452A/R472A were prepared, and steady-state kinetics for the sugars D-Glc, 6-deoxy-D-Glc, and D-xylene (lacking the exocyclic O6 group) and D-xylene (lacking the exocyclic C6–O6 group) was determined. For the wild-type enzyme (His6-rP2Ox), the Michaelis constant is at least one order of magnitude lower for D-Glc, which can form the proposed hydrogen bonds, than for 6-deoxy-D-Glc and D-xylene (K_m = 0.82 mM ± 0.03, 7.84 mM ± 0.63, and 24.0 mM ± 2.5, respectively). Abolishing the interaction with O6 by removal of one or both of the proposed hydrogen bond donors/acceptors resulted in significantly increased K_m values for the substrate n-Glc, whereas the corresponding data for the two substrates 6-deoxy-D-Glc and D-xylene were affected to a much smaller extent. Exchanging Asp452 with Ala had a more severe effect on the K_m values determined (data for His6-D452A obtained for D-Glc, 6-deoxy-D-Glc, and D-xylene were 104 mM ± 11, 68.5 mM ± 25, and 118 mM ± 14, respectively) than when Arg472 was exchanged (His6-R472A: K_m = 11.5 mM ± 3.9, 5.77 mM ± 1.55, and 9.05 mM ± 1.14, respectively, for these three substrates). The double mutation in His6-D452A/R472A resulted in an increase of >20-fold in the K_m values for D-Glc (K_m = 173 mM ± 13) as compared with the wild-type enzyme, indicating significantly weakened binding of the substrate in the active site. Michaelis constants for the substrates 6-deoxy-D-Glc and D-xylene were again less affected by these double mutations (K_m = 38.9 mM ± 11.5 and 74.2 mM ± 14, respectively).

Thus, the principal difference between C2ox and C3ox appears to be the possibility of one or two additional protein interactions made by O6 in the C2ox mode (e.g. to Arg472 Nε and Asp452 Oδ2), interactions that are not possible in the C3ox orientation. The additional interaction with Asp452 is enabled by the open conformation of the substrate loop where Asp452 has moved considerably closer to the active site compared with what is observed for the closed loop conformation in the nsP2OxACT complex (11). The hydrogen bonds donated by Thr169 to the flavin N5 atom when the substrate loop is in the closed conformation as in the acetate complex, may play a role in destabilization of the semiquinone during the oxidative half-reaction and disfavoring reduction by increasing the cost of protonating N5 during the reductive half-reaction. This is likely to promote the re-oxidation of the anionic hydroquinone to fully oxidized FAD. The Thr169 Oγ-FAD N5/O4 hydrogen bonds described here are also observed in human electron transfer flavoprotein (ETF) (29), where the enzyme variant T266M is pathogenic and appears to specifically affect the oxidative half-reaction between ETF and ETF-QO (30).

In the mutant, the loss of the hydrogen bond at N5 of the flavin, and the altered flavin binding increase the thermodynamic stability of the flavin semiquinone by 10-fold relative to the semiquinone of wild-type ETF. The mutation had relatively little effect on the reductive half-reaction of ETF catalyzed by sarcosine and medium chain acyl-CoA dehydrogenases that reduce the flavin to semiquinone. However, the K_cat/K_m of ETF-QO in a coupled acyl-CoA:ubiquinone reductase assay with oxidized αT266M ETF as substrate is reduced 33-fold; this decrease is due in largest part to a decrease in the rate of disproportionation of the αT266M ETF semiquinone catalyzed by ETF-QO. This lends indirect support to our earlier suggestion that the nsP2OxACT complex represents a structural state relevant for the oxidative half-reaction. A hydrogen bond between the flavin N5 atom and a backbone amide group is a recurrent theme in flavoenzymes (31). Only in a few reported cases is the donor a side-chain oxygen atom, such as in P2Ox, ETF, and medium chain acyl-CoA dehydrogenases. One may argue that a hydrogen bond between N5 and a backbone atom is less readily perturbed than when a side chain acts as hydrogen-bond donor. Thus, the suggested function of these threonine side chains to modulate differentially the redox potential of oxidized and reduced cofactor in the individual half-reactions may not be a general mechanism for flavin-dependent oxidoreductases.

Rationale for Product Outcome for Substrates Oxidized at C2—The P. gigantea P2Ox has been reported to oxidize some substrates at C2 preferentially, namely D-Glc, D-allose, D-galactose, 6-deoxy-D-Glc, D-gentiobiose, α-D-glucopyranosyl fluoride, and 3-deoxy-D-Glc (10). Based on the binding of 2FG to

Crystal Structure Complex of P2Ox with 2FG

Comparison of Ligand Binding in H167A2FG and nsP2OxACT—In nsP2OxACT, the active site exposes several hydrophobic side chains to the catalytic site: Ala171, Leu361, Phe454, Phe474, Val546, the Cβ-Cγ moiety of Arg472; and polar residues include Thr169, Gln448, Asn903, and His548. In His6-H167A2FG, the active site is more spacious and slightly less hydrophobic. This is due to the rearrangement of the substrate loop where Asp452 is brought into the active site, and the hydrophobic 154FSY456 segment is removed from the active site. In the closed loop conformation of nsP2OxACT, the side chain of Phe454 is positioned in the active site to interact closely, through van der Waals interactions, with the bound acetate molecule. Judging from the superimposition of His6-H167A2FG and nsP2OxACT, it is evident that the closed loop conformation is made incompatible with carbohydrate binding by the steric hindrance of Phe454 (Fig. 3C).

Furthermore, when the loop is closed (Fig. 3A), Thr169 adopts a rotamer conformation that results in the possibility of hydro-

gen bonds between the Thr side chain and the flavin N5 and O4 atoms (Thr169 Oγ1–N5, 3.0 Å; Thr169 Oγ1–O4, 2.9 Å). However, when the substrate loop is swung out and Phe654 removed from the active site, as observed in His6-H167A2FG, the Thr169 side chain has a different rotamer and forms no direct interactions with the flavin ring (Fig. 3B). By comparing unliganded and liganded active-site structures of the His6, H167A, Two important observations are noted: first, that the loop is open and well ordered in both, and second, that the Thr169 side chain adopts the same rotamer conformation, i.e. the conformation typically observed when the loop is open. Hence, the rotamer preference of Thr169 appears to depend solely on whether the substrate loop is open or closed and not on whether sugar substrate is bound or not.

We have proposed previously that the loop conformation observed in the acetate complex represents an active-site structure occurring during the oxidative half-reaction (11). The hydrogen bonds donated by Thr169 to the flavin N5 atom when the substrate loop is in the closed conformation as in the acetate complex, may play a role in destabilization of the semiquinone during the oxidative half-reaction and disfavoring reduction by increasing the cost of protonating N5 during the reductive half-reaction. This is likely to promote the re-oxidation of the anionic hydroquinone to fully oxidized FAD. The Thr169 Oγ-FAD N5/O4 hydrogen bonds described here are also observed in human electron transfer flavoprotein (ETF) (29), where the enzyme variant T266M is pathogenic and appears to specifically affect the oxidative half-reaction between ETF and ETF-QO (30).

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His\textsubscript{e}-H167A, it should be possible to rationalize the observed substrate preferences of P2Ox. For regioselective oxidation of substrate at either C2 or C3 by P2Ox or the C2–O2 or C3–O3 loci must be positioned and oriented appropriately with respect to the catalytic His\textsuperscript{548}–Asn\textsuperscript{593} pair. For most P2Ox substrates, the structural details of His\textsubscript{e}-H167A\textsubscript{PEG} explains the observed product outcome and, to some extent, the associated kinetic parameters.

The substrates oxidized at C2 all have in common that they contain an intact C2 hydroxyl functionality and that the carbohydrate structure is compatible with binding in the C2\textsubscript{ox} mode and/or incompatible with C3\textsubscript{ox}. D-Glc is most likely the preferred substrate (C3\textsubscript{ox} mode, we can expect that D-Glc will be able to bind in a similar way, if not identical. By rotating the glucose ring about an axis defined, see above, as shown in Fig. 4, the C3–O3 locus is transformed exactly onto the original position of C2–O2, and thus, becomes suitably positioned for oxidation.

D-xylose lacks the exocyclic C6–O6 group but nevertheless perfoms well as substrate for oxidation at C2 by P2Ox, albeit with a high \( K_m \) value (\( k_{\text{cat}} = 30 \text{ s}^{-1}; K_m = 30 \text{ mM}; k_{\text{cat}}/K_m = 1.0 \text{ mM}^{-1} \text{s}^{-1}; 7 \)). D-Galactose has the C4 hydroxyl group in the axial position. It is oxidized at C2 but performs rather poorly as substrate for P2Ox, e.g. 2-deoxy-D-Glc (data not shown), and therefore, the catalytic impairment is expected to involve a hydride-transfer mechanism (34). In this scenario, the protocol for the flavin is transformed exactly onto the original position of C2–O2, and thus, becomes suitably positioned for oxidation.

Rationale for Product Outcome for Substrates Oxidized at C3—Some substrates are selectively oxidized at C3 by P2Ox, e.g. 2-deoxy-D-Glc as well as methyl \( \beta \)-D-glucopyranoside and methyl \( \beta \)-D-galactopyranoside (10). As discussed above, 2FG is bound preferentially in the C3\textsubscript{ox} mode, which is consistent with the C2 position being substituted by fluorine, making this position unsuitable to react in 2FG. Similarly, 2-deoxy-D-Glc (\( k_{\text{cat}} = 55 \text{ s}^{-1}; K_m = 51.5 \text{ mM}; k_{\text{cat}}/K_m = 1.07 \text{ mM}^{-1} \text{s}^{-1} \)) lacks the C2 hydroxyl group, thus favoring C3 as substrate. In methyl \( \beta \)-D-glycosides (methyl \( \beta \)-D-Glc, \( k_{\text{cat}} = 45 \text{ s}^{-1}; K_m = 289 \text{ mM}; k_{\text{cat}}/K_m = 0.16 \text{ mM}^{-1} \text{s}^{-1} \)) and methyl \( \beta \)-D-Gal, \( k_{\text{cat}} = 1.0 \text{ s}^{-1}, K_m = 33 \text{ mM}; k_{\text{cat}}/K_m = 0.03 \text{ mM}^{-1} \text{s}^{-1} \)) (10), O1 is replaced by an \(-\text{O-CH}_3\) group. An extra methyl group at C1 as in methyl \( \beta \)-D-Glc and methyl \( \beta \)-D-Gal appears to be incompatible with the C2\textsubscript{ox} mode due to severe steric clashes with the peptide backbone of Val\textsuperscript{546} (distance methyl carbon, Val\textsuperscript{546} O < 1.5 Å). In C2\textsubscript{ox} mode, methyl-\( \beta \)-D-Gal has the additional disadvantage due to apparent steric hindrance between the axial galactosyl O4 atom and Thr\textsuperscript{169} O\textsuperscript{1}.

DISCUSSION

The presence of a firmly bound acetate molecule in the active site of nsP2Ox (11) revealed a similar relative position of an acetate carbohydrate oxygen to the catalytic center (the His–Asn pair and the FAD N5 atom) as that observed in CDH for the O1 hydroxyl group of a cellubiosein lactam inhibitor (32). Thus, based on the close relationship between the structures of P2Ox (11, 12) and the flavoprotein domain of CDH (33), the reaction mechanism of P2Ox is expected to involve a hydride-transfer mechanism (32, 11). In analogy with CDH, which oxidizes the reducing-end glucosyl moiety of cellubiose at C1, His\textsuperscript{548} in P2Ox (His\textsuperscript{549} in CDH) is likely to act as a general base to abstract the O2 hydroxyl proton accompanied by transfer of the C2 hydrogen as hydride to the re face of the flavin N5 (11). This hypothesis is supported by the steady-state kinetic parameters for His\textsubscript{e}-H548N (Table 1) where the catalytic turnover for His\textsubscript{e}-H548N is drastically reduced, whereas \( K_m \) remains only slightly affected. The Asn replacement of the catalytic His\textsuperscript{548} does not introduce structural changes beyond the substituted amino acid (data not shown), and therefore, the catalytic impairment of the His\textsubscript{e}-H548N mutant may be explained fully by its function, thus confirming the critical role of His\textsuperscript{548} in catalysis and stabilization of the transition state.

Besides providing substantial structural evidence for a hydride-transfer mechanism for cellubiose oxidation by CDH, we also acknowledged the shortcomings of crystal-structure analysis in evaluating the temporal sequence of events during hydride transfer (32). However, the relative timing of OH and CH bond cleavage in a hydride-transfer mechanism by GMC oxidoreductases has been discussed in detail recently (34, 35). For choline oxidation by choline oxidase from *Arthrobacter globiformis* (34, 35), the authors propose that hydride transfer is facilitated by the formation of an alkoxy species resulting from the removal of the substrate hydroxyl proton before hydride transfer to the flavin, thus implicating a non-concerted mechanism of hydride transfer (34). In this scenario, the protonated histidine (equivalent to His\textsuperscript{548} in P2Ox) would serve to...
stabilize the alkoxide species (35). A similar mechanism featuring an asynchronous transition state has been suggested earlier for methanol oxidase (36).

The possibility of an alkoxide species in CDH and P2Ox has not been investigated. However, the mechanism suggested by Gadda and co-workers (34, 35) implies that, because the role of the active-site histidine is redefined to stabilize the alkoxide species, another, yet unknown, catalytic base for the proton abstraction must exist. There is no crystal structure available for choline oxidase to argue against the hypothesis at this point, and it is difficult to make a direct comparison of the different enzymes, because, for CDH and related GMC enzymes, similar experiments have not been carried out. However, Gadda and co-workers (34, 35) report a 60-fold reduction in \( k_{\text{cat}} \) for the alanine replacement of the active-site histidine residue in choline oxidase with an associated 17-fold increase in \( K_m \). For the overall reaction, the equivalent mutation in CDH results in a 5,000-fold reduction in \( k_{\text{cat}} \), producing a catalytically incompetent enzyme, with an essentially unchanged \( K_m \) value using cellulbiose as substrate (37). The equivalent replacement has not been made in P2Ox, but as shown in this work, the asparagine replacement of His548 results in a 46,000-fold reduction in \( k_{\text{cat}} \) and a 62,000-fold reduction in specificity constant \( (k_{\text{cat}}/K_m) \). Conceivably, the active-site histidine residue may have different functions in different enzymes and, thus, makes generalization difficult.

For the majority of monosaccharide substrates, catalysis by \( T. \) \( \text{multicolor} \) P2Ox proceeds by oxidation at position C2 exclusively, and thus the enzyme active site discriminates effectively against C3 oxidation \( (i.e. \) oxidation at the carbohydrate C3 position), suggesting that some structural determinants responsible for discrimination of C2 versus C3 oxidation should be possible to identify. The fact that, in the absence of the substrate loop, \( \Delta \)-Glc could be modeled in position for both C2 and C3 oxidation (the two binding modes being related by a simple 180° rotation) to give a near identical set of protein-sugar interactions led us to suggest that the principal discriminating factors are likely to reside in the dynamic substrate loop (11).

The substrate analog 2FG was first used in soaking and co-crystallization experiments with wild-type P2Ox from the natural source to obtain a complex.\(^4\) The substrate loop appeared to be ambiguous in the wild-type complex such that two alternative conformations were present, one closed conformation that corresponds to the original nsP2Ox\( \text{ACT} \) Complex (11), and one open conformation that resembles that of the His\(_{548}\)-H167A\( \text{2FG} \) complex. This apparent disorder affects the 2FG ligand, which displays lower occupancy and less well defined electron density. By replacing the wild type by the H167A mutant, high ligand occupancy was obtained in the active site (Fig. 4A), and the substrate loop becomes fully ordered in the open conformation. This confirms that the open loop conformer is indeed relevant for binding of monosaccharide electron-donor substrate, whereas the closed loop conformation observed in the acetate complex is likely to represent a state required for binding of the second substrate, i.e. physiologically relevant electron-acceptor substrates such as \( \text{O}_2 \). It should be noted that several attempts were made to obtain a complex of P2Ox liganded with \( \Delta \)-Glc but without success.

The binding mode of 2FG is identical in the nsP2Ox and His\(_{548}\)-H167A active sites, corresponding to a glucose molecule oriented for oxidation at C3 rather than C2 (data not shown). Thus, the C3ox mode of binding in His\(_{548}\)-H167A is not due to the mutation, but more likely due to the fluorine atom at C2 of 2FG. The ability of P2Ox to oxidize 2FG at C3 is limited due to the highly electronegative fluorine atom that creates an electron-withdrawing effect, preventing electrons to transfer as hydride.

The reason for the difference in degree of disorder between the His\(_{548}\)-H167A and wild-type complex appears to originate from the ability of His\(_{548}\)-H167A to form a more stable complex with 2FG than is the case for wild-type P2Ox.

Due to removal of the histidyl–FAD bond, the redox potential of H167A is some 30 mV lower than that of the wild type (Table 2). A decrease in FAD oxidative power is observed, as shown by the decrease in turnover of \( \Delta \)-Glc from 50 to 9 s\(^{-1} \), as well as a decrease in reduction rate, \( k_{\text{cat}} \), by 2FG from 0.0664 to 0.000027 min\(^{-1} \). Thus, we show that 2FG is indeed a substrate, albeit slow, for P2Ox, in that it can reduce both wild-type and H167A P2Ox, which implies that P2Ox is able to oxidize \( \Delta \)-Glc at the C3 position. The slower turnover of 2FG by H167A compared with nsP2Ox explains why it is possible to trap a complex that displays well defined electron density for both ligand and substrate loop.

If assuming that the substrate loop conformation observed here for the C3ox mode of 2FG is true also for the C2ox binding mode, and that both binding modes are structurally valid for \( \Delta \)-Glc, the following should apply. Based on the modeling of the C2ox orientation, the discriminating determinant between the two binding modes is the position of the O6 hydroxyl group which, in the C3ox orientation is unable to make any favorable interactions with the protein. In the C2ox mode we predict that O6 will be able to interact with either Asp\(^{452} \text{O}^{\prime\prime} \) or Arg\(^{472} \text{N}^\ast \), or both. The possibility of an interaction with Asp\(^{452} \text{O}^{\prime\prime} \) is the result of the observed loop rearrangement. Whereas the shortest edge-to-edge distances of the side chains of Phe\(^{454} \) and Tyr\(^{456} \) in the nsP2Ox\( \text{ACT} \) and the monosaccharide in the His\(_{548}\)-H167A\( \text{2FG} \) complex are merely 1.5 and 2.9 Å, respectively, the shortest edge-to-edge distances between the Phe\(^{454} \text{F}\) and Tyr\(^{456} \text{F}\) side chains and the 2FG molecule in His\(_{548}\)-H167A\( \text{2FG} \) are 9 and 13 Å, respectively. This effectively excludes Phe\(^{454} \) and Tyr\(^{456} \) as possible discriminating determinants against C3ox of \( \Delta \)-Glc. As the substrate loop rearranges to allow binding of electron-donor substrate \( (i.e. \text{aldopyranose}) \), Asp\(^{452} \) is brought into the active site as a potential discriminator in the mechanism of regioselectivity. In addition, the importance of Asp\(^{452} \) and Arg\(^{472} \) for substrate binding and catalysis were confirmed by kinetic studies on mutant enzyme variants where these residues were replaced by alanine.

Differences in the position of the flavin ring as a result of removing the covalent histidyl–FAD linkage are likely to be responsible for the increase in \( K_m \). When comparing the position of the flavin ring and the 2FG molecule in the His\(_{548}\)-H167A

\(^4\) M. Kujawa, H. Ebner, C. Leitner, B. M. Hallberg, M. Prongjit, J. Sucharitakul, R. Ludwig, U. Rudsander, C. Peterbauer, P. Chaiyen, D. Haltrich, and C. Divne, unpublished results.
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and nsP2Ox complexes, we observe the following. In nsP2Ox, the covalent bond between His$^{167}$N$^c$ and FAD C8M results in a local shift of flavin atoms C5A, C6, and C7 of the dimethylbenzoid ring of 0.4 to 0.5 Å toward the monosaccharide binding site (data not shown). As a result, the 2FG molecule in the nsP2Ox is displaced by 0.4 Å in the same direction. Due to local disorder in the substrate loop of the nsP2Ox complex, caution should be exercised when evaluating the precise details of protein-ligand interactions. However, the most pronounced discrepancy between P2Ox$^{2FG}$ containing covalently and non-covalently attached FAD occurs at the exocyclic function where differences in atomic positions of 0.8 and 0.7 Å are observed for C6 and O6, respectively. Comparison of unliganded and 2FG-bound H167A shows virtually identical positions of the FAD cofactor, thus ruling out that the shift in position of the flavin ring is due to the substrate. Thus, covalent flavinylation of P2Ox via His$^{167}$ is not an absolute requirement for catalysis but necessary to refine the active site for optimal substrate binding and for modulating the redox properties of the flavin cofactor accordingly.

Lowering of the $E^\circ_m$ value as a result of removing the covalent histidyl–FAD bond has also been reported for vanillyl-alcohol oxidase (38). In this case, the H422A mutant (corresponding to H167A in P2Ox) displayed a 60% lower $K_m$ value and an 11-fold reduction in $k_{cat}$ compared with that of the wild type. A comparison of the unliganded structures of wild-type and H422A vanillyl-alcohol oxidase did not reveal any significant positional or conformational differences of cofactor or protein atoms, which is in agreement with the marginal effect on $K_m$ reported for the mutant. In P2Ox, however, a 5-fold increase in $K_m$ is observed as a result of eliminating the histidyl–FAD bond, which should correlate with the displacement of the cofactor and substrate observed in our structures.

It has also been suggested that the presence of a positive charge close to the dimethylbenzoid moiety of the flavin cofactor would serve to raise the redox potential (31), which is in agreement with a decrease in $E^\circ_m$ upon elimination of the histidyl–FAD bond. Moreover, the effect of enzyme flavinylation on flavin redox potential has been investigated extensively for p-cresol methylhydroxylase (39, 40). In these studies, the $E^\circ_m$ values of various FAD analogs in covalently and non-covalently bound states were determined. The results showed that the $E^\circ_m$ values of covalently bound FAD analogs were systematically higher compared with the noncovalently bound counterparts.

The action of oxidoreductases is of wide biological interest. Although studied extensively, the apparent difficulty to obtain crystal structures of ligand complexes for the GMC family of FAD-dependent oxidoreductases, together with the general complexity and versatility of flavoenzyme catalysts, has long complicated detailed mechanistic investigations. In this work, we addressed the mechanism of substrate specificity in general, and regioselectivity in particular, to shed light on P2Ox action. A stable, highly occupied and well ordered complex of the catalytically impaired H167A variant of P2Ox with 2-fluoro-β-glucose adds new insight into GMC oxidoreductase action, and a detailed structural basis for further functional and mechanistic investigation.

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