Crystal Structure of Glucooligosaccharide Oxidase from Acremonium strictum

A NOVEL FLAVINYLATION OF 6-S-CYSTEINYL, 8α-N-HISTIDYL FAD

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Glucooligosaccharide oxidase from Acremonium strictum has been screened for potential applications in oligosaccharide acid production and alternative carbohydrate detection, because it catalyzes the oxidation of glucose, maltose, lactose, cellobiose and cellobiono-1,5-lactam to 1.55- and 1.98-Å resolution, respectively. Unexpectedly, the protein structure demonstrates the first known double attachment flavinylination, 6-S-cysteinyl, 8α-N1-histidyl FAD. The FAD cofactor is cross-linked to the enzyme via the C6 atom and the 8α-methyl group of the isoalloxazine ring with Cys130 and His173, respectively. This sugar oxidase possesses an open carbohydrate-binding groove, allowing the accommodation of higher oligosaccharides. The complex structure suggests that this enzyme may prefer a β-D-glucosyl residue at the reducing end with the conserved Tyr429 acting as a general base to abstract the OH1 proton in concert with the H2 hydride transfer to the flavin N5. Finally, a detailed comparison illustrates the structural conservation as well as the divergence between this protein and its related flavoenzymes.

Sugar oxidases and dehydrogenases that catalyze carbohydrate oxidation to the corresponding lactones are of considerable commercial importance as potential diagnostic reagents and industrial biocatalysts. For example, glucose oxidase (GOX)§ is widely used in analytical biochemistry and in the food industry (1). A search of the BRENDA enzyme data base (2) reveals that most of these enzymes are specific for a variety of mono- and disaccharides, and only a few enzymes are highly selective for oligosaccharides. These include galactose oxidase, cellobiose dehydrogenase (CDH), and glucooligosaccharide oxidase (GOOX).

GOOX from Acremonium strictum was screened with the aim of identifying enzymes with potential applications in oligosaccharide acid production and alternative carbohydrate assays (3). It is a monomeric glycoprotein with a covalently linked FAD and catalyzes the oxidation of a variety of carbohydrates with the concomitant reduction of molecular oxygen to hydrogen peroxide. A screening of more than 50 carbohydrates and derivatives showed that D-glucose, maltose, lactose, and cellobiose are good substrates. In addition, this enzyme can react with malto- and cellobio- and glucooligosaccharides, and hence the name of this novel oxidase. The broad substrate specificity of GOOX, particularly toward oligosaccharides, suggests that it may have great potential applicability.

To facilitate further characterization and potential industrial use of A. strictum GOOX, we have cloned the encoding gene, which is composed of a 25-residue signal peptide and a 474-residue mature protein (4). Although GOOX shows a similar substrate specificity as GOX, CDH, and pyranose oxidase (POX), it shares no sequence similarity with them. However, GOOX displays significant sequence homology to the FAD-binding domain (F domain) of berberine bridge enzyme-like proteins including three other sugar oxidases, a red alga hexose oxidase (ChEOX), a tobacco nectar protein (nentin, NINECS), and a sunflower defense protein (HaCHOX) (5–7). Interestingly, although NINECS catalyzes oxidation of glucose, and GOOX, ChEOX and HaCHOX catalyze the oxidation of glucose, maltose, lactose, and cellobiose, the protein sequences of their substrate-binding domains (S domains) are quite diverse and apparently lack conserved carbohydrate-interacting residues. Moreover, structural and mutational studies demonstrated a consensus histidine for flavinylation in the F domain of berberine bridge enzyme-like proteins. Unexpectedly, mutation of the putative FAD-linking residue in GOOX, His430, to alanine, serine, cysteine, or tyrosine does not abolish the covalent FAD attachment (4). To gain structural insight into the FAD incorporation, substrate specificity, and catalytic mechanism, we have determined the GOOX structure in the absence, or presence of a product analog, 5-amino-5-deoxy-cellobiono-1,5-lactam (ABL).

MATERIALS AND METHODS

Recombinant protein was expressed using the vector pPICZαA in Pichia pastoris KM71, and then isolated by a Toyopearl Phenyl-650 column with elution of a gradient of 2.5–0 ml ammonium sulfate (4). Yellow protein crystals were grown in 25% polyethylene glycol mono-
methyl ether 550, 10 mM ZnSO₄, and 0.1 M MES (pH 6.5), with a combination of 2 μl of reservoir solution and 2 μl of protein solution (35 mg/ml). Crystals appeared and reached their final dimensions in a month at 295 K. Only very few batches of recombinant proteins produce crystals of high quality. The high-resolution datasets were collected using the synchrotron radiation at the beamlines BL122B2 at SPring-8 (Harima, Japan) and NW12 at Photon Factory (Tsukuba, Japan). The data were processed using the HKL2000 program (8). The crystals belong to the P2₁2₁2₁ space group with 1 monomer/asymmetric unit. Because of a lack of fresh crystals, the frozen native crystals were used for preparation of the ABL derivative by soaking crystals for 30 min in a reservoir solution containing 60 mM ABL. The x-ray data were collected on a RAXIS IV area detector with a Rigaku RU-300 rotating anode (MSC).

Heavy atom screening yielded one platinum derivative (K₂PtI₄) and sufficient phase information was derived from single-wavelength anomalous dispersion. Four Pt positions were identified with SHLEXD (9) and refined with SOLVE (10). The initial phase was improved significantly by direct method phasing refinement using OASIS (11) before density modification. An initial model with 248 polyalanine residues was built by RESOLVE (10). The electron density was improved greatly by fragment extension with OASIS, so that a complete model with side chains was built by ARP/wARP (12). The structure then underwent straightforward refinement against native data to 1.55 Å resolution using CNS (13). The structure of the ABL derivative was solved by molecular replacement using the refined coordinates mentioned above as the initial model. Statistics for data collection and refinement are summarized in TABLE ONE. More than 90% of the residues are in the favored regions of Ramachandran plot, with the remaining in the additional allowed regions except for Thr³²⁷ and Arg³⁴⁵, because of hydrogen bond interactions. Crystals with better quality were grown in a reservoir containing 60 mM ABL, and accommodate the FAD cofactor between them.

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**TABLE ONE**

| Data collection and refinement statistics | Native | K₂PtI₄ | ABL |
|-----------------------------------------|--------|--------|-----|
| Data collection                          |        |        |     |
| Unit cell (Å)                           | 53.1, 90.9, 111.7 | 53.8, 91.5, 113.0 | 53.7, 91.1, 112.3 |
| Wavelength (Å)                          | 0.7    | 1.0718 | 1.5418 |
| Resolution range (Å)                   | 50–1.55 | 50–2.0 | 50–1.98 |
| Total observations                      | 786,463 (27,250) | 486,389 (40,996) | 124,905 (7,832) |
| Unique reflections                      | 77,613 (4,395) | 37,715 (3,628) | 35,571 (3,144) |
| Completeness (%)                       | 98.1 (95.9) | 97.8 (95.6) | 90.5 (81.8) |
| I/σ(I)                                  | 30.1 (3.3) | 25.8 (9.2) | 15.0 (3.5) |
| Rmerge (%)                              | 6.1 (27.0) | 5.1 (14.3) | 8.9 (29.2) |
| Refinement                              |        |        |     |
| Resolution range (Å)                   | 50–1.55 (1.56–1.55) | 50–1.98 (1.99–1.98) |
| Refections (F > 0 σ₁)                  | 77,613 (1,471) | 35,571 (628) |
| Rmerge (%) for 90% data                | 18.1 (24.8) | 20.0 (28.4) |
| Rmerge (%) for 10% data                | 20.0 (25.4) | 24.0 (30.2) |
| Root mean square deviations            |        |        |     |
| Bond lengths (Å)                        | 0.005 | 0.006 | 1.36 |
| Bond angles (°)                         | 1.39 |        |     |
| Mean B value (Å²)                      |        |        |     |
| | 3,757 Protein atoms                  | 17.6 | 3,757 Protein atoms | 30.2 |
| | 53 FAD atoms                         | 12.9 | 53 FAD atoms | 19.4 |
| | 515 water molecules                  | 32.2 | 287 water molecules | 31.2 |
| | 23 ABL atoms                         |        |        | 36.0 |

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**RESULTS AND DISCUSSION**

**The Overall Structure**—The current model contains five linker residues and the 474-residue mature GOOX. The protein is composed of two distinct domains (Fig. 1). The F domain recruits the N-terminal residues 1–206 and the C-terminal residues 421–474 and folds into two (α+β) subdomains. A small subdomain, residues 1–90, comprises a central four β-strands (β₁–β₄) with the strand order 1, 2, 4, and 3, and with β₁ running antiparallel to the other strands. The β-sheet is sandwiched by three helices (αA, αA’, and αB), in which there is one disulfide bond between Cys₅ of the αA helix and Cys⁵⁰ of the αB helix. The second subdomain contains five antiparallel β-strands (β₅–β₉) with the strand order 5, 6, 9, 7, and 8 and is surrounded by five α-helices (αC, αD, αD’, αI, and αK). These two subdomains are packed against each other and accommodate the FAD cofactor between them.

Furthermore, the S domain (residues 207–420) is composed of a large seven-strand antiparallel β-sheet (β₁₀–β₁₆) with the strand order 13, 10, 12, 11, 15, 16, and 14 and is flanked by five helices (αE–αI). This domain is positioned over the isoalloxazine ring of the FAD cofactor and constitutes most of the carbohydrate-binding groove. Two N-glycosylation modifications were detected in the S domain. The electron density showed clearly one ordered sugar residue each linked to Asn³⁰⁵ and Asn³⁴¹, which was assigned as N-acetyl-d-glucosamine based solely on fitting to the electron density.

**A Novel Flavinylation**—Unexpectedly, the FAD cofactor is cross-linked to the enzyme at two attachment sites (Fig. 2A). One is the S⁷ atom of Cys³⁰⁵ bound to the C⁵ atom of the isoalloxazine ring, whereas the other is the N⁷ atom of His³⁷⁰ bound to the 8α-methyl group (6S-cysteinyl, 8α-N1-histidyl FAD). The ADP-ribityl moiety is embedded in the F domain and is completely solvent-inaccessible. The adenine and
ribose groups form eight hydrogen bonds with two water molecules and
the backbone atoms of Ala285, Gly306, and Val360 (Fig. 2B). The negatively
carged pyrophosphate group interacts with a constellation of back-
bone NH groups of Gly67, Gly68, Gly69, His70, Ser71, Gly134, and Gly137. I n
charged pyrophosphate group interacts with a constellation of back-

analogue ABL (18). The four types of flavinylation at the FAD-interacting segments are highlighted in cyan. The two major FAD-interacting segments are highlighted in magenta. The lactam moiety of ABL is bound in the −1 subsite. The lactam C1′ atom, which corresponds to the site of oxidation in cellobiose, binds in front of the flavin N5 with a distance of 3.35 Å and an angle with the N5′–N6′ atoms of 105°. It should be noted that like for the CDH complex (21), the lactam C1′ and O1′ atoms are almost perfectly aligned with the flavin N5 and C4′, respectively.

The lactam O1′ atom interacts with Tyr429O (2.78 Å) and Gln384N (2.14 Å), and the endocyclic NH makes close contacts with the Tyr72O (3.34 Å) (Fig. 3B). The lactam O2′ group forms hydrogen bonds with Thr129O (2.84 Å), Arg245N (3.13 Å), and the isoalloxazine O (3.23 Å), the equatorial O H3 with Arg245N (3.15 Å) and Glu330O (2.86 Å), and the OH with Tyr72O (2.88 Å) and Tyr300O (3.67 Å). Thus, a total of nine hydrogen bonds are formed at the −1 subsite. In contrast, the −2 glucosyl moiety of ABL forms only one direct protein-carbohydrate hydrogen bond between OH3 and Glu555O (3.30 Å). Additionally, the OH2 and OH4 groups form two and three water-mediated hydrogen bonds, respectively. Tyr300 and Trp351 stack on the pyranose ring with an interplanar distance of 4.2–4.3 Å. This type of stacking interaction is very common in protein-carbohydrate recognition. The ABL binding does not induce any significant conformational change except for the side chain of Glu247.

On the basis of the bound ABL molecule described above, a variety of carbohydrate molecules were modeled manually in the carbohydrate-binding groove, and the model was subjected to energy minimization with CNS. α-Glucose is the only monosaccharide substrate for GOOX (3). Simulation of the complexes shows that other hexoses and derivatives form either fewer hydrogen bonds or unfavorable contacts with the residues surrounding the substrate-binding groove. The axial OH2 group in allose and the absence of an OH2 in

FIGURE 1. Stereo view of the GOOX structure. The protein consists of a FAD-binding (F) and a substrate-binding (S) domain, colored in red (helix) and green (strand). The intermediate analogue ABL (magenta), the cofactor FAD (black), the linking residues His70 and Cys130, and the glycosylated Asn305 and Asn341 are displayed as ball-and-stick representations. The two major FAD-interacting segments are highlighted in cyan. The hydrogen bonds between the backbone of one residue at the N terminus of the β strand (Val195 in GOOX) with the adenine N1 and N6 are strictly conserved.

Substrate Specificity—ABL resembles cellobionolactone, the oxidation product of cellobiose, with replacement of the endocyclic O5 with an NH group (Fig. 3). This replacement suppresses the spontaneous hydrolysis of ABL to cellobionic acid. ABL is the only inhibitor against GOOX activity found to date. Approximately 50% of glucose oxidation activity was lost in the presence of 25 mM ABL. In addition, ABL has been shown to be an inhibitor for CDH. The structure of CDH in complex with this inhibitor has been solved and used to delineate a reaction mechanism for CDH (21). In the complex structure presented here, ABL is identified by its strong electron density that correlates well with the shape of d-glucopyranosyl rings, and is firmly embedded on the si face of the isoalloxazine ring (Fig. 3A). The lactam moiety of ABL is bound in the −1 subsite. The lactam C1′ atom, which corresponds to the site of oxidation in cellobiose, binds in front of the flavin N5 with a distance of 3.35 Å and an angle with the N5′–N6′ atoms of 105°. It should be noted that like for the CDH complex (21), the lactam C1′ and O1′ atoms are almost perfectly aligned with the flavin N5 and C4′, respectively.

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2-deoxy-\(\alpha\)-glucose and of the exocyclic \(\text{CH}_2\text{OH}\) in \(\alpha\)-xylose would entail a small number of hydrogen bonds. The axial \(\text{OH}_2\) in mannose, the axial \(\text{OH}_4\) in galactose, and the equatorial \(\text{NH}_2\) in glucosamine would cause unfavorable contacts with Gln\text{353}, Trp\text{351}, and Arg\text{245}, respectively. Modification at the C\text{1}, C\text{2}, and C\text{6} positions such as phosphorylation and \(N\)-acetylation would cause steric hindrance because of lack of appropriate space. Like CDH and galactose oxidase (21, 22), GOOX possesses an open carbohydrate-binding groove, so that the non-reducing end of the glucose residue can stick out into the solvent and be exposed on the protein surface (Fig. 3C). This explains why the enzyme is able to utilize oligosaccharides as good substrates (4). In contradistinction, the crystal structures of GOX

FIGURE 3. The carbohydrate-binding site. A, stereo view of the 2F_o – F_c electron density map for the intermediate analogue ABL contoured at 1.2σ level. B, stereo view of the interaction networks between ABL and GOOX. Hydrogen bonds are shown as green dashed lines. There are eight direct hydrogen bonds between the protein and the –1 sugar, but only one hydrogen bond between the protein and the –2 sugar (see “Results and Discussion” for a detailed explanation). C, molecular surfaces of GOOX. The protein surface is colored for electrostatic potential from –20 k_BT (red) to 20 k_BT (blue), reflecting its pI value of 4.3–4.5 (3). The FAD cofactor is colored in black, and the modeled cellohexaose is in green. The open carbohydrate-binding groove explains why oligosaccharides are good substrates.
and POX have revealed a “size-exclusion mechanism,” in which the shape of the active site cavity is such that the enzyme can only accept monosaccharides (23, 24).

Catalytic Mechanism—Most of the FAD-dependent oxidases and dehydrogenases are proposed to function via a hydride transfer rather than by a carbanion mechanism (25, 26). The pH optimum of 10 for GOOX implies that a tyrosine residue may serve as a general base (3). Based on the ABL binding, the substrate cellulose was modeled into the active site. The reducing end C1 atom of the –1 sugar binds in front of the flavin N5 at a distance of 2.86 Å and an angle with the N5–N10 atoms of 113°. These values are in agreement with those typically observed for flavoenzymes, and appropriate for a direct hydride transfer (25, 26). However, no appropriate general base for carbanion formation can be identified. The hemiacetal OH1 group of the β-anomer at the –1 subsite could interact with Tyr429O (3.11 Å), Gln384N (3.69 Å), and the isoolxalizine O4 (3.11 Å), whereas that of the α anomer may make close contacts with Thr529O (3.17 Å) and the isoolxalizine O4 (3.25 Å). The distance between the reducing end H1 of the β-anomer and the flavin N5 is 2.13 Å, whereas that for C1H of the α to the flavin N5 is 2.64 Å. Therefore, GOOX may preferentially oxidize the β-anomer possessing an equatorial hydroxyl group, with the conserved Tyr429 acting as a general base.

As is common for flavoenzymes, the reaction mechanism of GOOX consists of two half-reactions (Scheme 1). The reductive half-reaction is involved in the oxidation of the free reducing end β-D-glucosyl residue to glucono-1,5-lactone by hydride transfer to the N5 atom, probably initiated by proton abstraction from the OH1 group by Tyr429. The lactone product is spontaneously hydrolyzed to gluconic acid. In the oxidative half-reaction, regeneration of the oxidized FAD by molecular oxygen yields hydrogen peroxide. Water-mediated hydrogen bonds between Asp355 and Tyr429 suggest that Asp355 may assist in proton transfer by lowering the pKa value of Tyr429 through a water molecule. Interactions between Gln384 and the reducing end OH2 group suggest that Gln384 may contribute to position the substrate and facilitate proton abstraction by Tyr429. His338, Tyr426, and the backbone NH of Tyr444 may be involved in stabilization of the anionic form of the reduced flavin. A large water-filled channel above the carbohydrate-binding groove and toward the flavin moiety may act as an entry point for the second substrate, molecular oxygen (Fig. 3C).

Structural Conservation and Divergence in the PCMH Superfamily—A structural homology search by DALI (27) revealed that GOOX displays significant structural similarity to *Zea mays* cytokinin dehydrogenase (ZmCKX), *Brevibacterium sterolicum* cholesterol oxidase 2 (BsCOX2), *Pseudomonas putida* p-cresol methyldihydroxyase (PpPCMH), *Penicillium simplicissimum* vanillyl-alcohol oxidase (PsVAO), and *Escherichia coli* β-lactate dehydrogenase (EcDLDH), with root mean square deviations of 2.8 Å (414 C atoms with 16% sequence identity), 3.5 Å (398 Ca atoms with 10% identity), 3.5 Å (398 Ca atoms with 16% identity), 3.4 Å (404 Ca atoms with 13% sequence identity), and 3.6 Å (369 Ca atoms with 12% identity), respectively (28–32). The structural homologies of these flavoproteins seem to suggest a divergent evolutionary relationship, and thereby are defined as the PCMH superfamily because the crystal structure of PpPCMH was the first one reported (33). However, the relative orientation between the F and S domains does vary greatly across the different structures. The F domain also shares structural homology to *E. coli* uridine diphospho-N-acetylglucosamine reductase (EcMurB), *Oligotropha carboxidivorans* CO dehydrogenase (OcCODH), and *Bos taurus* xanthine oxidase (BtXOX) (34–36).

The FAD binding modes are one of the least conserved properties among flavoproteins, and the flavinylation sites are often located in poorly conserved regions (18, 37). Although these PCMH members share a similar F domain, they contain different FAD binding types. In addition to the novel flavinylation in GOOX (6-S-cysteiny1, 8α-N1-histidy1 FAD), the FAD cofactor utilizes its 8α-methyl group cross-linking to His105 in ZmCKX and His121 in BsCOX2 (8α-N1-histidy1 FAD), to His122 in PsVAO (8α-N3-histidy1 FAD), and to Tyr384 in PpPCMH (8α-O-tyrosyl FAD). The remaining members bind FAD non-covalently.

The F domain is much more superimposable than the S domain, perhaps because of distinct substrate binding but a similar FAD recognition mechanism across the enzymes. The eight β-strands (β2–β9, 60 structurally equivalent residues) of the F domain, overlay within 0.8–1.4 Å root mean square deviations (Fig. 4A). Interestingly, these members utilize many main-chain atoms for the FAD binding, and hence the sequences of the FAD-interacting residues are divergent, whereas their spatial positions are convergent in four regions (Figs. 1 and 4B). The first region is located in the loop between the β3 and β4 strands and shares similar features with the "P-loop," which is highly conserved in nearly all nucleotide-binding proteins and responsible for interaction with the pyrophosphate group of the cofactor NDP moiety (38). The second region involves the short αD helix, and the third region is located at the N terminus of the β9 strand. It is worth noting that the hydrogen bonds between the
adenine N1 and N6 and the backbone of one residue at the N terminus of the β9 strand (Val195 in GOOX) are strictly conserved, even in EcMurB, OcCODH, and BtXOX. The last region is at the C-terminal tail, and the residues utilize the side chains for the cofactor binding.

In contrast, the seven β-strands (β10–β16) of the S domain, particularly the β11 and β14–β16 stands, varies greatly across the different structures. These diverse four β-strands make up the majority of the substrate-binding site (Figs. 1 and 4B) and result in the highly divergent architectures of the substrate-binding site needed for the various different substrates ranging from a small lactate to the bulky cholesterol and long chain oligosaccharides. The closed conformations of ZmCKX, BsCOX2, PpPCMH, and PsVAO, limit the size of the substrate-binding

FIGURE 4. Structural conservation and divergence in the PCMH superfamily. A, stereo view of structural superposition of the β2–β9 strands of GOOX (red), ZmCKX (green), BiCOX2 (blue), PpPCMH (black), PsVAO (yellow), EcDLDH (cyan), and EcMurB (pink). The β-strands correspond closely, whereas the α-helices and surface-exposed loops diverge significantly. & structure-based sequence alignment. The accession codes are listed in the right column. The secondary structure elements for GOOX are labeled (ss). The number of residues present in gaps is indicated in parentheses. The flavinylation sites are colored in red and indicated by *. Residues making close contacts with FAD are shaded in magenta and those for the conservative hydrophobic core are in yellow. The substrate-binding residues are shaded in cyan, and the putative conserved sugar-binding residues in GOOX, NINECS, HaCHOX, and CcHEOX are in green. The diverse S domains of these sugar oxidases were aligned on the basis of the secondary structure prediction and the conservative hydrophobic core.
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pocket and thus result in a \"size-exclusion mechanism\" (28–31). In contradistinction, GOOX has an open carbohydrate-binding groove to accommodate larger oligosaccharides.

Convergent Evolution in FAD-utilizing Sugar Oxidases—According to the structural fold, flavoenzymes have been classified into many superfamilies (37). Interestingly, folding topology does not correlate with enzyme function. For example, GOOX and BsCOX2 belong to the PCMH superfamily, whereas GOX, POX, CDH, and BsCOX1 belong to the glutathione reductase superfamily. Location of the active center at the re face of the isoalloxazine ring is generally conserved within the glutathione reductase superfamily, whereas that of the PCMH members is on the si side. Therefore, these structures provide elegant examples of convergent evolution, where starting from different ancestral folds, the same FAD-assisted flavinylation to optimize the active site for enhancement of its oxidative power.

Interestingly, even starting from a similar structural fold, sugar oxidases evolve some dissimilar residues for interaction with the common substrates. Within the glutathione reductase superfamily, GOX, POX, and CDH catalyze the oxidation of glucose, and share a similar FAD-binding fold facilitating flavinylation to optimize the active site for enhancement of its oxidative power.

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