Crystal Structure of Glucooligosaccharide Oxidase from — A Novel Flavinylation of 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD

Glucooligosaccharide oxidase (GOOX) 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 cello- and malto-oligosaccharides (Fig. 1) [1]. Here we have determined the GOOX structure in the absence, or presence of a product analog, 5-amino-5-deoxy-cellobiono-1,5-lactam (ABL) (**BL12B2**) (Fig. 2(a)) [2].

Unexpectedly, the FAD cofactor is cross-linked to the enzyme at two attachment sites (Fig. 2(b)). One is the S^{γ} atom of Cys^{130} bound to the C^{6} atom of the isoalloxazine ring, while the other is the $N^{\delta 1}$ atom of His^{70} bound to the 8α-methyl group (6-*S*-cysteinyl, 8α-*N*1-histidyl FAD). Five types of covalent flavinylation have been identified up to the present and flavinylation has been shown to be an autocatalytic process [3]. The four types of flavinylation at the 8α-methyl group cross-link to His ($N^{\delta 1}$ and $N^{\varepsilon 2}$), Tyr (O^{η}) and Cys (S^{γ}), whereas an unusual 6-S-cysteinyl modification has been observed only in the FMN cofactor of trimethylamine dehydrogenase and its homologues. Thus, GOOX possesses a novel form of covalent flavinylation; it is the first example of 6-*S*-cysteinyl FAD and the first double covalent linkage identified to date. The His^{70} and Cys^{130} mutants suggest that the covalent attachment is able to enhance the redox potential of the flavin [1].

ABL is firmly embedded on the *si* face of the isoalloxazine ring without induction of any significant conformational change except for Glu^{247} (Fig. 3(a)). A variety of carbohydrate molecules were then modeled manually in the substrate-binding groove. D-glucose is the only monosaccharide substrate for GOOX. Simulation of the complexes shows that other hexoses and derivatives form either fewer hydrogen bonds or unfavorable contacts with the surrounding residues. GOOX possesses an open carbohydrate-binding groove, which explains why the enzyme is able to utilize oligosaccharides as good substrates (Fig. 3(b)). The substrate cellobiose was modeled into the active site, suggesting that GOOX preferentially oxidizes the β anomer with the conserved Tyr^{429} acting as a general base. As is common for flavoenzymes, the reaction mechanism of GOOX consists of two half-reactions (Fig. 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 N^{5} atom, probably initiated by proton abstraction from the OH^{1} group by Tyr^{429}. 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.

According to the structural fold, flavoenzymes have been classified into many superfamilies [4]. Interestingly, folding topology does not correlate with enzyme function. For example, GOOX and *Brevibacterium sterolicum* cholesterol oxidase 2 (BsCOX2) belong to the *p*-cresol methylhydroxylase (PCMH) superfamily, whereas glucose oxidase,

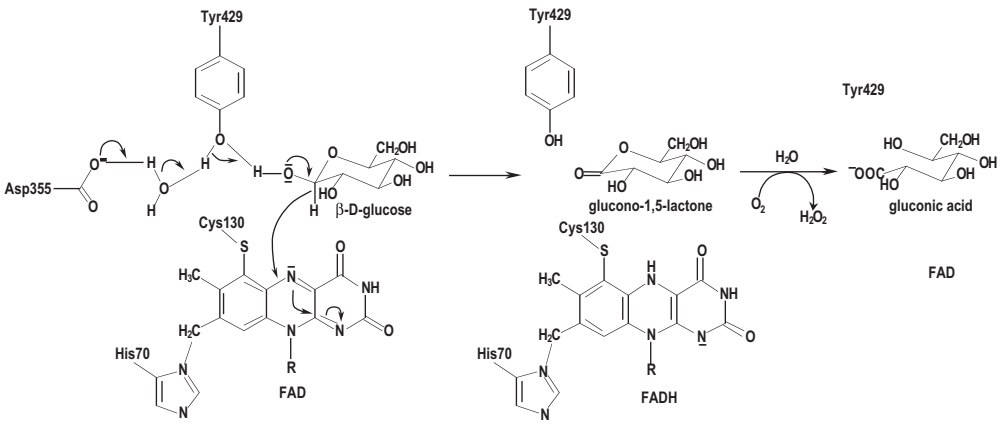

Fig. 1. The proposed catalytic mechanism for GOOX.

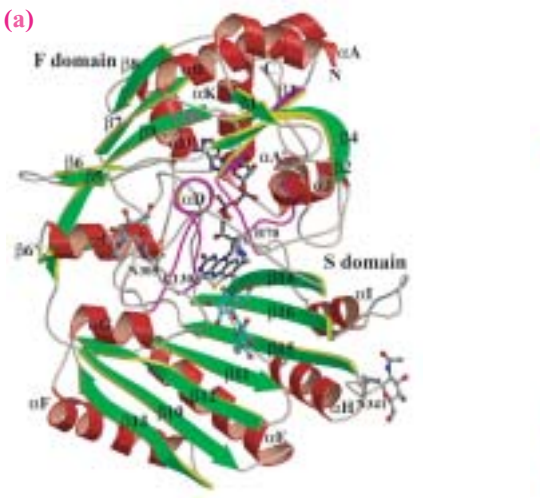

Fig. 2. (a) The GOOX structure. The protein consists of a FAD-binding (F) and a substrate-binding (S) domain, colored in *red* (helix) and *green* (strand). The intermediate analogue ABL (*cyan*), the cofactor FAD (*black*) and the linking residues His70 and Cys130, and the glycosylated Asn305 and Asn341 are displayed as *ball-and-stick* representations. The two major FAD-interacting segments are highlighted in *magenta*. **(b)** The 2Fo - Fc electron density map for the FAD cofactor and the covalently bound His70 and Cys130 contoured at 2σ level. The density map demonstrates the first known double attachment flavinylation, 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD.

cellobiose dehydrogenase and BsCOX1 belong to the glutathione reductase (GR) superfamily. Location of the active center at the *re* face of the isoalloxazine ring is generally conserved within the GR 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 glucose (or cholesterol) oxidation is achieved through opposite flavin faces within distinct substrate-binding sites. Even starting from a similar structural fold, the sugar oxidases have evolved a similar FAD-assisted oxidation mechanism but different substrate recognition, resulting in distinct binding affinities to the mono- and di-sacchardes, with *Km* values ranging from 50 μM to 30 mM.

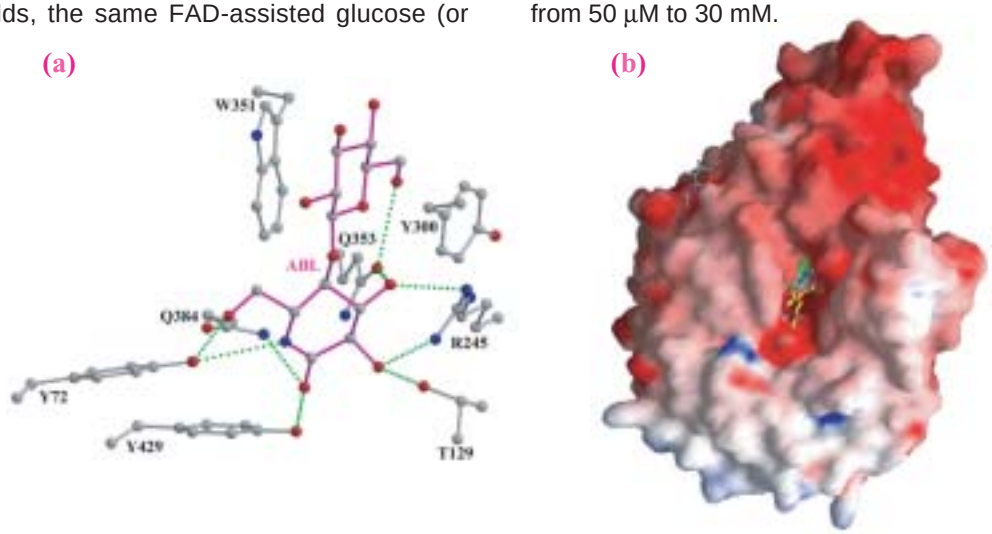

Fig. 3. **(a)** The interaction networks between ABL and GOOX. Hydrogen bonds are shown as green dashed lines. **(b)** Molecular surfaces of GOOX. The protein surface is colored for electrostatic potential from -20 k_BT (*red*) to 20 k_BT (*blue*), reflecting its pI value of 4.3-4.5. The FAD cofactor is colored in *green*, while the modeled cellobiose is in *yellow*. The open carbohydrate-binding groove explains why oligosaccharides are good substrates.

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