The vast repertoire of carbohydrate oxidases: An overview

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ABSTRACT

Carbohydrates are widely abundant molecules present in a variety of forms. For their biosynthesis and modification, nature has evolved a plethora of carbohydrate-acting enzymes. Many of these enzymes are of particular interest for biotechnological applications, where they can be used as biocatalysts or biosensors. Among the enzymes catalysing conversions of carbohydrates are the carbohydrate oxidases. These oxidative enzymes belong to different structural families and use different cofactors to perform the oxidation reaction of CH-OH bonds in carbohydrates. The variety of carbohydrate oxidases available in nature reflects their specificity towards different sugars and selectivity of the oxidation site. Thanks to their properties, carbohydrate oxidases have received a lot of attention in basic and applied research, such that nowadays their role in biotechnological processes is of paramount importance. In this review we provide an overview of the available knowledge concerning the known carbohydrate oxidases. The oxidases are first classified according to their structural features. After a description on their mechanism of action, substrate acceptance and characterisation, we report on the engineering of the different carbohydrate oxidases to enhance their employment in biocatalysis and biotechnology. In the last part of the review we highlight some practical applications for which such enzymes have been exploited.

1. Introduction

Carbohydrates are biomolecules widely available in all life forms and fulfill a plethora of functions. Thanks to their structural features, carbohydrates can arrange in long polymers and, differently from nucleotides and amino acids, they can easily form branches. This structural flexibility is provided by the different binding modes possible between carbohydrates, which can form glycosidic bonds between various positions upon a dehydration reaction.

For synthesis, modification or degradation purposes, many enzymes have carbohydrates as their natural substrates. Upon enzymatic catalysis, carbohydrates can be transferred to acceptors, modified in their structure, polymerised, or hydrolysed. Among the enzymes acting on sugars, carbohydrate oxidases have been extensively studied and explored for their possible applications in biotechnology. The oxidation reaction (Fig. 1a) occurs on a CH-OH or CH-NH (EC 1.1.3.x or EC 1.5.3.x) bond and results in the formation of a double bond (C=O or C=N, respectively). This reaction is dependent on a redox cofactor hosted in the enzyme’s structure and on the availability of molecular oxygen, used to regenerate the cofactor, with the concomitant production of hydrogen peroxide. Oxidation is typically directed towards specific positions of the sugar ring. Such regioselectivity is often absolute and makes carbohydrate oxidases attractive biocatalytic tools because such a high degree of regioselectivity is difficult to achieve by chemical means. The most frequently observed regioselectivities for carbohydrate oxidases are C1, C2 and C6 oxidations (Fig. 1b). Except for exquisite regioselectivity, carbohydrate oxidases typically have a very defined substrate acceptance profile, acting on a limited number of carbohydrates. The exact substrate specificity and regioselectivity relies on the unique active site architecture of each carbohydrate oxidase.

The physiological role of many carbohydrate oxidases is still debated, as the oxidation reaction often overlaps with the chemistry performed by carbohydrate dehydrogenases. While dehydrogenases capture the electrons generated upon oxidizing the carbohydrate using a coenzyme, typically NAD(P)⁺, a flavin or a cytochrome, oxidases reduce molecular oxygen to form hydrogen peroxide. Flavoprotein (carbohydrate) oxidases often can also use alternatively electron acceptors. Vice versa, flavin-containing carbohydrate dehydrogenases typically display a low activity with oxygen as electron acceptor, acting as (inefficient) oxidases. This reflects that fact that the microenvironment around the flavin cofactor can promote (oxidases) or demote (dehydrogenases) the oxygen reactivity of the reduced flavin cofactor. In fact,
Carbohydrate oxidases are used as O₂ scavengers in environments functional intracellularly. Disregarding their natural functions, carbohydrate oxidases and other oxidases in fungi is proposed to be coupled to the action of peroxidases and peroxigenases, which rely on hydrogen peroxide to initiate lignin degradation (van Hellemont et al., 2006). Recent insights into intracellular signalling has shown that hydrogen peroxide can also serve a role as signalling molecule (Sies, 2017). This may explain why, although rare, some oxidases are known to be functional intracellularly. Disregarding their natural functions, carbohydrate oxidases have been found to be very handy biocatalytic tools. Carbohydrate oxidases are used as O₂ scavengers in environments where oxygen can be detrimental to storage or processing of food. The enzymes can also be used as reporters for sugar-specific biosensors and even find use in the synthesis of specific compounds, such as vitamins, antioxidants and bulk compounds for the chemistry market. This review will provide an overview of all known carbohydrate oxidases in the context of biotechnological applications.

Carbohydrate oxidase is a term used to describe enzymes that are (1) active on the same class of substrates, carbohydrates, and (2) can use molecular oxygen as mild oxidant. The number of characterised carbohydrate oxidases has grown significantly in the last few decades and these are listed in Table 1. From the known characteristics, several different classes can be distinguished (Fig. 2). A first distinction can be made based on the cofactor used by the enzyme to perform the oxidation reaction. So far, carbohydrate oxidases contain either copper as redox cofactor, or a flavin cofactor. Copper dependent oxidases (Daou and Faulds, 2017) make use of a single copper ion, which is bound in the protein core, held in place by surrounding residues. The best-known member of this class is the fungal galactose oxidase. All copper-containing carbohydrate oxidases belong to the same structural family. The known flavin-dependent carbohydrate oxidases contain a tightly, sometimes even covalently, bound FAD cofactor. Flavoprotein carbohydrate oxidases can be classified according to the structural fold into two distinct families: the glucose-methanol-choline oxidase (GMC) family (Sützl et al., 2019) and the vanillyl-alcohol oxidase (VAO) family (Fraaije et al., 1998). Known members of the GMC-like oxidases are glucose oxidase and pyranose oxidase, which are among the most studied carbohydrate oxidases. VAO-like examples are the chito- and glucos-oligosaccharide oxidases. A further distinction is based on the presence and number of covalent bonds present between the FAD cofactor and the protein part of the specific oxidase (Heuts et al., 2009). This special feature will be discussed for each FAD-dependent oxidase, as it is relevant for their properties and engineering.

In Table 1, all described carbohydrate oxidases are listed. By following this table – presented in a chronological order according to the year of cloning – the reading of the following sections will be facilitated. In the next parts of the review, the enzymes are clustered according to their cofactor dependence and their three-dimensional structure. Such organisation has been chosen over substrate acceptance or organism of origin to avoid redundancy, as many carbohydrate oxidases belonging to the same type have been found in different organisms, and the substrate acceptance range is often very wide, or overlaps in many cases.

![Fig. 1.](image.png) (a) General reaction catalysed by carbohydrate oxidases acting on a CH-OH bond. (b) The numbering of carbons in carbohydrates.

2. Copper-dependent carbohydrate oxidases

2.1. Galactose oxidase

Among the copper-dependent oxidases acting on carbohydrates, the prototypical enzyme is certainly galactose oxidase. A vast amount of available literature makes this enzyme an ideal example to explain the features of this valuable biocatalyst and how it is different from the FAD-dependent oxidases. The enzyme has been known since the end of the 1950s (Cooper et al., 1959) and was found to be secreted by different fungi. While GAO is known by virtue of its reactivity towards galactose, it displays oxidase activity with a vast range of substrates, including oligosaccharides and even molecules other than carbohydrates. Galactose derivatives and galactose-containing oligosaccharides were tested, revealing high conversion rates (Avigad et al., 1962) such as in the case of D-galactosamine, lactose, raffinose, melibiose and stachyose. Other substrates include dihydroxyacetone (Zancan and Amaral, 1970) and benzyl alcohol derivatives (Whittaker and Whittaker, 2001). More recently, GAO was challenged with much larger galactose-containing polysaccharides. It was shown that it is active towards galactoglucomannan, galactomannan, arabino-galactan, arabinoxylan and xylglucan, for which even MDa size can be reached (Parikka et al., 2010). Even if the degree of activity was different for each of these polysaccharides, this study demonstrates the possibility to make use of GAO in new and different applications, such as the preparation of gelling agents both for food industry and as biopolymers precursors.

The structure of galactose oxidase corresponds to a monomer composed of three distinct domains (Fig. 4a), where the N-terminal domain contains a carbohydrate binding module used to recognise substrates, while the central domain contains the active site, hosting the copper ion. The copper ion interacts with three residues belonging to the central domain (two tyrosines and one histidine) and one other histidine residue belonging to a third domain (Ito et al., 1991). The third domain is the C-terminal part of the protein and hangs above the central domain, penetrating in it such a fashion that the histidine residue lies above the reactive center. Another feature typical of GAO is the presence of a cross-link between one of the tyrosines involved in the copper binding and a nearby cysteine. Such bond is formed post-translationally (Rogers et al., 2000) and is necessary to the reaction cycle of GAO. The active site is isolated from the solvent thanks to a tryptophan residue strategically positioned just beside the cross-linked residues, in the second-coordination sphere of the active site (Rogers et al., 2007).

The catalytic cycle of GAO (Himo et al., 2000; Humphreys et al., 2009) follows a ping-pong mechanism which starts with the binding of the substrate in front of the copper ion (Fig. 4e). Upon abstraction of the hydroxyl proton of the substrate by action of a tyrosine residue, a second tyrosine, involved in the cross-link with cysteine, sequester the proton of the carbon atom. The oxidised product is released from the active site, while molecular oxygen takes its place in front of the copper ion. Oxygen is reduced in two steps by the previously mentioned tyrosines and the newly formed hydrogen peroxide released, allowing for a new reaction cycle to begin. Molecular oxygen is responsible for the regeneration of the reduced enzyme. Despite the vast number of possible substrates, GAO is extremely regioselective, and acts specifically...
| Enzyme name                     | Abbreviation | Cofactor | Fold family | Source organism                      | Melting temperature [°C] | Size of the monomeric unit [kDa] | Reported best substrate(s)                                                                 | PDB structure(s) | Year of publication |
|--------------------------------|--------------|----------|-------------|--------------------------------------|--------------------------|----------------------------------|-----------------------------------------------------------------------------------------|------------------|---------------------|
| L-gulonolactone oxidase         | GUO          | FAD      | VAO         | Animal, Rattus norvegicus, insects    | 50                       | 50                               | L-gulono-1,4-lactone                                                                 | 1GAL             | 1988                |
| Glucose oxidase                 | GOX          | FAD      | GMC         | Fungi, Aspergillus niger, Penicillium spp., insects | 63                       | 80                               | D-glucose, fructose, amino acids, lactose, maltotriose, lactose, lactose, maltose, methyl α-D-glucosylophonide, methyl β-D-glucosylophonide, maltose, maltotriose | 3DJD, 3DJE       | 1989                |
| Fructosyl-amino acid oxidase    | FAO          | FAD      | VAO         | Yeast, fungi, Aspergillus niger, bacteria | 50–60                     | 57                               | maltose, maltooligosaccharides, lactose, cellobiose and glucose                        | 1ZG6, 2AKX       | 1991                |
| Glucosidogalactoside oxidase    | GOO          | FAD      | VAO         | Fungi, Acremonium sp, Sarocladium striatum | 60–65                     | 60                               | D-glucose, D-galactose, D-galactosamine, dihydroxyacetone, raffinose, melibiose, stachyose, methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, large polysaccharides | 1GOF, 2ER, 2JKX  | 1992                |
| Galactose oxidase               | GAO          | Cu       | VAO         | Fungi, Fusarium graminearum, Fusarium spp. | 60                       | 68                               | D-galactose, D-galactosamine, dihydroxyacetone, raffinose, melibiose, stachyose, methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, large polysaccharides | 1T2L, 2IGO, 3K4L | 1996                |
| Pyranose oxidase                | POX          | FAD      | GMC         | Fungi, Phlebiopsis gigantea           | 60                       | 65                               | D-glucose, D-galactose, fructose, amino acids, lactose, maltoligosaccharides             | 4RSL, 5T1E       | 2002                |
| Hexose oxidase                  | HOX          | FAD      | VAO         | Plant, Chondrus crispus              | 55                       | 45                               | xylitol, sorbitol                                                                      | 1ZG6, 2AKX       | 2000                |
| Ascari-1,4-lactone oxidase      | ALO          | FAD      | VAO         | Yeast, Saccharomyces cerevisiae, proteus Leishmania donovani | < 50                     | 60                               | fructosyl-peptides                                                                      | 4RSL, 5T1E       | 2002                |
| Sorbitol oxidase                | SO           | FAD      | VAO         | Bacteria, Streptomyces sp, H 7775     | 55                       | 45                               | xylitol, sorbitol                                                                      | 1ZG6, 2AKX       | 2000                |
| Xylitol oxidase                 | XO           | FAD      | VAO         | Bacteria, Streptomyces sp, IKD-472    | 55                       | 45                               | xylitol, sorbitol                                                                      | 1ZG6, 2AKX       | 2000                |
| Lactose oxidase                 | LAO          | FAD      | VAO         | Fungi                                | 50                       | 65                               | D-glucose, D-maltose, D-lactose, β-glucose, α-glucose, fructose, amino acids, lactose, maltose, maltoligosaccharides | 4RSL, 5T1E       | 2002                |
| Fructoyl peptide oxidase        | PPO          | FAD      | VAO         | Fungi                                | 50–60                     | 50                               | fructose-1,6-diol, fructose, amino acids, lactose, maltose, maltoligosaccharides           | 4RSL, 5T1E       | 2002                |
| Carbohydrate oxidase            | CHO          | FAD      | VAO         | Plant, fungi                         | 70                       | 57                               | fructose-1,6-diol, fructose, amino acids, lactose, maltose, maltoligosaccharides           | 4RSL, 5T1E       | 2002                |
| Glucosolactone oxidase          | GLO          | FAD      | VAO         | Fungi, Streptomyces sp, H 7775        | 60                       | 60                               | fructose-1,6-diol, fructose, amino acids, lactose, maltose, maltoligosaccharides           | 4RSL, 5T1E       | 2002                |
| Chitooligosaccharide oxidase     | ChitO        | FAD      | VAO         | Fungi, Fusarium graminearum           | 63                       | 52                               | N-acetyl-lactosamine, lactose, maltose, lactose, maltose, maltoligosaccharides             | 4RSL, 5T1E       | 2002                |
| Alditol oxidase                 | AldO         | FAD      | VAO         | Bacteria, Streptomyces sp, Acidothermus cellulolyticus | 65                       | 45                               | fructose-1,6-diol, fructose, amino acids, lactose, maltose, maltoligosaccharides           | 4RSL, 5T1E       | 2002                |
| Radical copper oxidase           | Gbx          | Cu       | VAO         | Bacteria, Streptomyces lividans       | 67                       | 67                               | glycolaldehyde, α-glucose, D-glucose, D-galactose, glycerol                            | 5LXZ             | 2015                |
| Xylooligosaccharide oxidase      | XyO          | FAD      | VAO         | Fungi, Myceliophthora thermophila C1   | 65                       | 65                               | xylose, xylotriose, xylotetraose                                                       | 5K8E             | 2016                |
| Raffinose oxidase               | RaOx         | Cu       | VAO         | Fungi, Penicillium rubens, Colletotrichum graminicola | > 50                     | 70                               | fructose, melibiose, stachyose, D-galactose, glycerol, glycolaldehyde dimer              | 3RJ8             | 2004                |
| Cellobiose oxidase              | PbBBE1       | FAD      | VAO         | Plants                               | 54                       | 54                               | cellobiose, lactose, α-mannitol, D-sorbitol                                             | 6E04             | 2018                |
| Mannitol oxidase                | MOX          | –        | VAO         | Gastropods                           | 54                       | 54                               | cellobiose, lactose, α-mannitol, D-sorbitol                                             | 6E04             | 2018                |
| Mannitol oxidase                | UAO          | FAD      | VAO         | Plants, Citrus sinensis              | 62                       | 62                               | D-galacturonic acid, D-gluconic acid, polygalacturonic acid                             | 6E04             | 2020                |

PDB structures here listed can refer to homologs from organisms other than the main reference reported. More structures are available for some entries, for example presenting bound ligands. The structural formulas of the carbohydrates mentioned as substrates in this table are shown in Fig. 3.
Fig. 2. Scheme of carbohydrate oxidase classification based on the type of cofactor and its binding mode.

Fig. 3. Some substrates of known carbohydrate oxidases.
on position C6 (Fig. 5). This has been confirmed by the incapability to convert L-galactose.

Since the first cloning experiments (McPherson et al., 1992) GAO has been found in different organisms, cloned and expressed in recombinant form (Faria et al., 2019), revealing different substrate preferences, such as in the case of GAO from *Fusarium sambucinum* (Paukner et al., 2015) for which melibiose is the substrate with highest catalytic efficiency. The optimal temperature for GAO activity in this case was found to be 30 °C and the enzyme quickly inactivated at 60 °C. Optimisation of the reaction conditions even allowed to increase the conversion of α-pyranoside to the aldehyde form thanks to the reaction with a water molecule (Parikka and Tenkanen, 2009).

Engineering of GAO has been attempted in numerous studies, taking advantage of the wide range of substrates that the enzyme accepts. Since the regio- and enantioselectivity of GAO is well documented and ascribed to the residues involved in the ligand binding (F194, W290, Y329, R330, Q406, F464 and Y495), directed evolution has been used to adapt its acceptance to secondary alcohols of high relevance as building blocks (Escalattes and Turner, 2008). Protein engineering has also been directed towards GAO specificity for polysaccharides, by generating fusions of GAO and carbohydrate binding modules different from the galactose-specific module normally present in GAO (Mollerup et al., 2016). Conversely, even glucose oxidase activity has been introduced in GAO, maintaining the specificity for oxidation at position C6 of the sugar ring, something which was not previously possible by using any known oxidase (Sun et al., 2002). Further engineering even led to the use of GAO as a tool to perform labeling of glycosylated proteins (Rannes et al., 2011) and to the introduction of fructose oxidase activity by mutagenesis of the pivotal residues Arg330 and Phe464 (Deacon et al., 2004) Also, to facilitate the use of GAO, several immobilisation strategies have been explored (Kanyong et al., 2017) and delivered highly stable enzyme formulations which remained active up to two weeks (Kondakova et al., 2007).

2.2. Other newly discovered copper-containing carbohydrate oxidases

Another copper-containing carbohydrate oxidase has been recently identified in the bacterium *Streptomyces lividans* (Chaplin et al., 2015). It is a copper-containing oxidase, which has been named GlxA after the coding gene, and is characterised by distinct spectroscopic features with respect to the previously described and well-known galactose oxidase. The activity of GlxA is expected to be towards position C6 and its best substrate is glycolaldehyde, but its substrate acceptance profile also includes α-glucose, D-galactose, and glycerol. No activity was observed for fructose, GlcNAc, D-glucuronic acid or disaccharides. Effects of GlxA impairment were evaluated in vivo demonstrating its role in glycan
synthesis.

The three-dimensional structure of GlxA resembles the organisation of GAO, and indeed the RMSD between the second domain of the two enzymes is very low (1.18 Å). The N-terminus of GlxA is involved in transmembrane interactions. Direct contacts are established between the copper ion and two histidines, two tyrosines and one water molecule. The different substrates acceptance with respect to GAO is likely caused by structural differences such as the lower accessibility of GlxA active site, the different residues composing the environment of the binding site and the different location of the carbohydrate binding module.

Raffinose oxidase is another example of a newly described copper-containing oxidase. This enzyme belongs to the same family of GlxA (Andberg et al., 2017), which includes relatively few characterised members at the moment (Yin et al., 2015). Raffinose oxidase (RaOx) from the fungus Colletotrichum graminicola displayed activity for the oligosaccharide raffinose ($K_m = 480$ mM), the disaccharide melibiose and for D-galactose to a lower extent. No activity was measured for other monosaccharides. NMR spectroscopy was used to determine the oxidation site of RaOx substrates, which again confirmed regioselective oxidation at C6.

The discovery of RaOx further expands the repertoire of GAO-like enzymes. Another raffinose oxidase was also found in *Penicillium rubens* and characterised ((Mollerup et al., 2019). Differently from the Colletotrichum homologue, stachyose is also a substrate. Stachyose is a tetrasaccharide, sharing the structure of raffinose but with an extra D-galactose residue.

While the physiological role of these oxidases is still not clear, it is suggested that they are involved in pathogenicity and in defence against fungi and plants. From a biotechnological perspective, the availability of natural variants of the same enzyme with different ranges of substrate acceptance makes them interesting targets of study. Also, while no engineering has been attempted on the above-mentioned carbohydrate oxidases, these novel enzymes open a new territory for exploring oxidase-based biotechnological applications. The biocatalytic features of GlxA and RaOx have been included in a discussion on the structural and functional properties of galactose oxidase-like enzymes (Yin et al., 2015). While the latest developments in this topic escape the scope of this review – having no data concerning the utilisation of recently found copper oxidases – we expect that members of this family of copper-containing carbohydrate oxidases could become important tools for biotechnology in a near future.

3. GMC-like flavin-dependent carbohydrate oxidases

3.1. Glucose oxidase

The GMC superfamily includes enzymes with various functions and substrate specificities, but that share a common fold and the presence of a FAD cofactor (Sützl et al., 2019). The GMC fold includes a FAD-binding domain, which comprises a Rossmann fold, and a substrate-binding domain (Fig. 4b) (Cavener, 1992). This last domain is the most variable one and is responsible for different substrate specificities in the various members of the superfamily. The FAD cofactor is typically tightly bound as prosthetic group. In a few cases, the flavin cofactor is even covalently bound to a nearby residue. Despite the variability in substrates acceptance, the reaction cycle of GMC-type carbohydrate oxidases is conserved, with first a reduction of the flavin cofactor by transfer of a hydride from the substrate and a subsequent reoxidation of the cofactor through using molecular oxygen as an electron acceptor (Wongnate and Chaiyen, 2013).

Several well-known carbohydrate oxidases are part of this superfamily of flavoproteins. Among these, glucose oxidase has received most attention. The first cloning of the gene encoding for GOX from *Aspergillus niger* was achieved at the end of the ’80s (Kriechbaum et al., 1989) and this discovery was shortly followed by the first GOX crystal structure (Hecht et al., 1993). GOX has been isolated from many fungal species such as *Penicillium* spp. and *Talaromyces flavus* (Kusai et al., 1960; Kim et al., 1990).

The molecular weight of GOX is 80 kDa for the monomer but the physiological arrangement is dimeric. The natural reaction catalysed by the enzyme is the oxidation at C1 of $\alpha$-glucose, yielding D-glucono-lactone and hydrogen peroxide (Fig. 6). Because of the hydrogen peroxide production, as in the case of galactose oxidase, a physiological role as weapon against other microorganisms has been proposed (Wong et al., 2008). As the enzyme is widely represented in fungal species, also an association with lignin degradation enzymes is suspected, by providing hydrogen peroxide to peroxidases. While the enzyme is active over a large pH range, product accumulation can result in lowering of the solution’s pH and inactivation of GOX (Bao et al., 2003).

The activity of wild type GOX from *A. niger* against substrates other than $\alpha$-glucose is quite low, with only a few exceptions such as 2-deoxy and 6-deoxy forms of $\alpha$-glucose (Wong et al., 2008). No activity for any oligo- or polysaccharide has been reported. This narrow substrate acceptance is in line with the elucidated structure of GOX which has revealed a rather occluded active site.

Engineering of GOX has been limited due to the fact that no expression of GOX has been established in a convenient bacterial expression system. Also, the lack of a crystal structure with a ligand, such as a substrate or product, complicates the design of mutant libraries. Recently, some engineering of GOX has been reported by using yeast as expression system. To make the enzyme accustomed to industrial conditions, in silico design was applied to develop protein variants that displayed higher heat resistance, improved tolerance to low pH and an extended half-life. The final variant was shown to yield higher gluconic acid titres and having a higher melting temperature (+8.5 °C) (Mu et al., 2019).

Cofactor engineering with GOX has also been performed. In a recent study, FAD was replaced with 7,8-dichloro-FAD (Tremey et al., 2014). This kind of replacement is particularly interesting for the GOX application as a bioelectrode. The engineered system is much less sensitive to molecular oxygen, which is typically responsible for loss of current in biosensing systems based on electrochemistry. Various other FAD derivatives have been incorporated in GOX with the aim to tune its properties towards a specific application. One other recent example is the incorporation of progesterone-FAD conjugates, in which the steroid is linked to the adenine moiety of FAD. Such modified GOX would allow a sensitive method to detect such steroids (Posthuma- Trumpie et al., 2007). The availability of protocols to prepare apo GOX is essential for these cofactor engineering approaches. While several protocols for this have been described, it remains challenging to prepare apo GOX in an efficient manner.

Immobilisation of GOX has been used to assess the conversion of sugars by oxidation at C3 position on equatorial hydroxyl groups.
3.2. Pyranose oxidase

The enzyme pyranose oxidase (POX) is also a GMC-type flavoprotein oxidase which catalyses the oxidation of pyranoses, including D-glucose, at position C2. For this reason, it is important not to confuse this enzyme, which is also called glucose 2-oxidase, with the above-described GOX (Mislovicová et al., 2010).

The reaction mechanism of POX matches the previously described ones, with release of a ketone and hydrogen peroxide. In-depth analysis of the catalytic mechanism of POX has revealed that the oxidase in unique in stabilizing an hydroperoxylavolin intermediate, caused by the timely proton transfer of an essential histidine residue (His548) and stabilised by residues composing the surrounding microenvironment (Wongnate et al., 2014). A recent study shed more light on the POX-catalysed carbohydrate oxidation step (Wongnate et al., 2019) which involves an initial hydride transfer from C2 of α-glucose to the flavin, followed by the involvement of the aforementioned histidine residue, which abstracts the remaining proton from the ketone form of the sugar.

After the initial discovery in Polyporus obtusus (Janssen and Ruelius, 1968), the enzyme was found in other fungi (Giffhorn, 2000) and orthologs are frequently discovered, finding new applications (Karmali and Coelho, 2011). While most described POXs represent secreted fungal enzymes, recently also bacterial POXs have been discovered (Zhang et al., 2018; Mendesa et al., 2016).

The first thorough characterisation of POX from Phlebiopsis gigantea was reported in the mid ’90s (Schafer et al., 1996) and revealed a tetrameric arrangement of the enzyme, a broad pH range and high thermostability. Substrates of POX were found to be α-glucose, L-sorbose, and D-xyllose. The crystal structure of a POX from a white-rot fungus, Peniophora sp., was obtained in 2004 (Bannwarth et al., 2004) and confirmed the oligomeric state. The structure also allowed a structural and mechanistic comparison with GOX and there are currently many structures available, including some with ligands bound (Fig. 4f), such as 2-fluoro-2-deoxy-α-glucose (PDB entry 2IGO) and 2-fluoro-galactose (4MOO). Among the most relevant differences with respect to GOX and other GMC-type enzymes is the presence of a covalent bond between the isoalloxazine ring of the FAD cofactor and a histidine residue His167 (Fig. 4c). The presence of this histidyl linkage increases the oxidation potential of the enzyme, as reported for members of the VAO family (Fraaije et al., 1999). The general architecture of the active site differs significantly from GOX. A major difference of POX is the presence of an active site loop which received much attention in follow-up mechanistic and engineering studies. These differences well explain the change in oxidation site with respect to GOX, as well as the wider substrate acceptance of POX. Still, both GOX and POX only accept monosaccharides due to their relatively well-buried active sites.

The mechanism of catalysis by POX has been investigated using kinetic studies and mutants of POX (Sucharitakul et al., 2010). While this work provided insights into the selectivity of POX for α-glucose by using deuterated analogues, further details on the regioselectivity were elucidated by using fluorinated versions of the substrate sugars (Tan et al., 2011). The crystal structure of the mutant H167A with a bound fluorinated analogue (PDB entry 3PL8) provided insights in the strict regioselectivity of the enzyme, which allows exclusive C2 oxidation. On the same research line, a few years later, mutants of POX from Trametes multicolor were characterised to understand their improved activity on D-galactose with respect to wild type enzyme (Spadiut et al., 2010; Tan et al., 2014). The effect of backbone relaxation caused by the mutations was remarkable. This study was relevant since it enables the use of an engineered POX as a catalyst for the oxidation of both the products of lactose hydrolysis, α-glucose, and D-galactose.

As we can see from the wide distribution of POX in white-rot fungi, the link to lignin degradation has also been suggested this oxidase. The importance of POX in providing H2O2 for the activity of peroxidases has been highlighted (Daniel et al., 1994) together with the role of the product D-glucosone in the synthesis of the cortical hormone (Baute and Baute, 1984).

Several enzyme engineering studies have been performed on POX. The goals of these engineering efforts vary from boosting the thermostability to improve activity towards specific substrates. A POX from Peniophora gigantea underwent directed evolution yielding a variant with an increase in optimum temperature by 6 °C and much higher conversion of D-xylose (60 fold), D-sorbose (69 fold) with respect to the wild type enzyme (Bastian et al., 2005). Similar experiments conducted on POX from Trametes multicolor resulted in an increased stability, which made the enzyme operative for long periods at 60 °C (Spadiut et al., 2009a, 2009b). To address the low affinity of POX for molecular oxygen and boost reactivity, a strategy which proved successful has been the combined use of POX and catalase. This in situ destruction of hydrogen peroxide and regeneration of oxygen was tested in the case of fructose production from sorbitol (Schneider et al., 2012). The newly discovered bacterial POXs may be attractive enzyme engineering targets because they allow facile expression in Escherichia coli, which is often problematic for fungal enzymes. Yet, a disadvantage of the bacterial homologs is the absence of a covalent FAD-protein linkage which may be detrimental for the operational or storage stability.

3.3. Fructosyl amino acid oxidase

Fructosyl amino acid oxidases (FAOs) are active on Amadori compounds, for which reason they are also known as Amadoriases (Horiuchi et al., 1989). Amadori compounds are carbohydrates that underwent a non-enzymatical glycation process. Glycation is the reaction between a sugar and an amino group. The two compounds react to form a Schiff base, with the coupled release of a water molecule. FAOs cleave the linkage between the carbohydrate and the amine. Since this is an oxidative reaction, they release the amine and an oxidized carbohydrate. Molecular oxygen is used as electron acceptor resulting in formation of hydrogen peroxide, and for this reason they can be classified as carbohydrate oxidases (Wu and Monnier, 2003). It should be pointed out that such reaction occurs on a CH-NH bond, rather than on CH-OF bonds, as it was in previous examples (Fig. 8).

FAOs have been characterised from fungal and bacterial sources. The bacterial ones were isolated from Corynebacterium sp. 2.4-1 (Horiuchi et al., 1989), Agrobacterium tumefaciens (Hirokawa and Kajiyama, 2002) and Pseudomonas aeruginosa (Saxena et al., 1996). The presence of these enzymes allows them to use fructosyl amino acids as carbon source. The Corynebacterium FAO is active on many D-fructosyl-L-amino acids. The Agrobacterium FAO displayed similar properties.
including a dimeric arrangement, substrate acceptance and relative instability (rapidly inactive at 40 °C). The *Pseudomonas* FAO has the special feature of releasing not the glucose product, but fructoseamine instead, revealing a different mode of oxidation. Fungi also seem a rich source of FAOs, which are also dimeric (Takabashi et al., 1997; Jong et al., 2002). Nevertheless, sequence comparison has revealed that the prokaryotic and eukaryotic proteins display a relatively low sequence identity. For this reason, even if the structure of the *Aspergillus fumigatus* FAO has been solved, no reliable structural models of the bacterial counterparts can be generated.

The general folding of the elucidated fungal FAO structure allows classifying it as member of the GMC superfamily. Still, the structural resemblance when comparing the FAO structure (PDB: 3DJD) with the structure of glucose oxidase (1GAL) is low (RMSD is only 3.4 Å). The first FAO structure unveiled an unusual mode of flavin cofactor binding: the FAD is covalently bound via a β-S-cysteinyl-FAD linkage to the nearby Cys335 (Collard et al., 2008). This sets the fungal FAOs apart from the bacterial FAOs, which harbour a non-covalently bound FAD cofactor. The role of the linking cysteine residue as well as the general folding of fungal FAOs were predicted even before the elucidated crystal structure, based on homology with sarcosine oxidase (a GMC oxidase). Other studies focused on investigating the active site of FAO, focusing on the role of a lysine residue close to the isoalloxazine ring of FAD (Collard et al., 2011). Based on mutagenesis and stopped-flow experiments, Lys53 was found to be critical for an efficient flavin reduction (the first half of the Amadoriase reaction).

When it comes to engineering of FAO, the bacterial enzyme from *Corynebacterium* has been engineered to increase its thermostability (Sakae and Kajiyama, 2003). Fungal FAO has been targeted by random and site-directed mutagenesis studies. Both approaches were directed towards increasing the specificity of the enzyme for fructosyl valine as a substrate. In the random mutagenesis approach, an increase in specificity was achieved at the expense of activity (Fujiiwara et al., 2006). The site-directed mutagenesis experiments (Miura et al., 2008) were based on a homology model (Miura et al., 2006). The role of an asparagine residue was discovered thanks to docking experiments and the tested mutants were indeed showing a higher specificity for fructosyl valine. Based on these results it should be possible to further develop FAO variants capable of acting on single fructosyl amino acids more efficiently than the wild type enzyme.

### 3.4. Fructosyl peptide oxidase

The substrates of fructosyl peptide oxidases (FPOs) are fructosyl peptides, in which fructose is linked to a peptide. The catalysed reaction is the same as FAOs. Thus, also FPOs can be regarded as Amadoriases. As in the FAO case, FPO acts on a CH-NH bond. FPOs have been reported mostly from fungi and share high homology in sequence with eukaryotic FAOs. They also have a similar size (50 kDa proteins) and a covalently bound FAD cofactor (Sakai et al., 1995). The crystal structure of FPO strongly resembles FAO, and the RMSD between the structure of FPO from *Eupenicillium terrenum* (PDB entry 4RSL) and FAO from *Aspergillus fumigatus* is only 1.7 Å. The first crystal structure of an FPO was published in 2015 (Gan et al., 2015) and revealed that FPOs have a larger entrance to the active site than FAOs, which allows to accommodate the peptide moiety of the substrates. Thanks to the comparison between a ligand-free and a fructosyl thiocetate-bound structure, it became clear how the elements defining the active site (loops and helices) are shorter than the topological equivalent parts of FAO, allowing the fructosyl peptides to bind. The residue involved in the cysteinyl linkage of FAD is Cys347 in the *E. terrenum* enzyme. Further structural studies on the FPO from *Phaeosphaeria nodorum* revealed details on the oxidase activity on fructosyl-α-N-valyl-histidine (Shimasaki et al., 2017). Specifically, an aspartate in position 54 has been recognised as a gatekeeper for oxygen entrance and its role is essential for an efficient oxidative half-reaction for regeneration of the oxidized flavin cofactor. Engineering strategies on FPO involved mutagenesis of an asparagine in position 56. This residue has previously been targeted to probe the relevance of Asp54 in oxidation (Shimasaki et al., 2017). Mutation of Asn56 causes a decreased reactivity with molecular oxygen, resulting in a variant not reactive with oxygen. This allows to use such variants as dehydrogenases by utilizing artificial electron acceptors, such as the dye 2,6-dichlorophenoldiphenol (Kim et al., 2012). To address the estimation of glycated haemoglobin, FPO was further engineered by saturation mutagenesis of the loop surrounding the active site of the enzyme (Ferri et al., 2013). This was possible by combining two loops from two different FPOs, from *Phaeosphaeria nodorum* and from *Coniochaeta* sp. NISL 9330. At the same time, the Asn56Ala substitution was also introduced to enhance the dehydrogenase activity of FPO.

FPOs can display specificity for both α-keto-amine and ε-keto-amine or just for α-keto-amine and this aspect has been studied in different fungal strains (Hirokawa et al., 2003a) by investigating the specific ratio of activity of the enzyme on different substrates. According to these findings it is possible not only to develop diagnostic tools capable of recognising glycated proteins in patient samples, but also to recognise the sites at which glycation occurs (Hirokawa et al., 2003b). These tools are relevant for the detection of spontaneously glycated haemoglobin in diabetes patients and, conversely, to monitor the efficiency of treatments (Yonehara et al., 2015). Engineering the specificity for the type of glycation has been also attempted in view of enhancing the screening of diabetes patients’ blood samples. Since the target for glycated haemoglobin detection is the ε-glycated fructosyl-lysyl-histidine, the aim (Shabbazmohammadi et al., 2019) was to increase the activity of FPO for this substrate while reducing the specificity for the ω-glycated fructosyl-histidine. Using a combination of in silico experiments (molecular docking and molecular dynamics) and testing of promising mutants in vitro, it was possible to increase the activity ratio FruValHis/Fru-Lys from 1.6 to 129, making glycated haemoglobin detection much easier.

### 4. VAO-like flavin-dependent carbohydrate oxidases

#### 4.1. Oligosaccharides oxidases and lactose oxidase

The carbohydrate oxidases belonging to the group of VAO-like flavoprotein oxidases share a common fold, which is different from the one we described in the previous part for GMC-like flavoprotein oxidases. The general structure (Fig. 4d) is once more divided in a FAD-binding domain and in a substrate-binding domain (Fraaije et al., 1998). The cofactor is deeply buried in the protein structure, but in this
case, it is always covalently bound to a histidine residue, which tethers to the C8 of the isoalloxazine ring. A second cofactor bond is often present between a cysteine and the C6 of the flavin cofactor. The substrate binding site is generally quite accessible to the solvent. As we will see this has some important implications in the kind of substrates accepted by these enzymes.

Glucooligosaccharide oxidase is a VAO-like oxidase which displays substrate specificity for mono-, oligo- and polysaccharides. For this reason, the active site is a large groove that goes from the protein surface to the isoalloxazine ring of the cofactor, allowing the binding of ligands having different size (Huang et al., 2005). Similar to the above-mentioned glucose oxidase, the regioselectivity is towards position C1. This means that the reducing end of a sugar chain is bound close to the reductase active flavin cofactor. Despite the structure being solved only in the mid-2000s, GOO was known since the beginning of the 90s, when it was first found in Acremonium strictum and recognised for its capability to convert oligosaccharides (Lin et al., 1991). The stability of GOO is highest at somewhat basic pH and the enzyme was found to be stable up to 50 °C (Fan et al., 2000). The GOO crystal structure was the first to show a bicovalent binding mode of FAD, via histidyl- and cysteinyl-flavin covalent bonds. Subsequent studies have demonstrated the essential role of both covalent bonds by generating enzyme variants through site-directed mutagenesis (Huang et al., 2008). When the single GOO mutants H70A and C130A were compared to the wild type GOO, their redox potential was found to be much lower and, therefore, also their flavin reduction rate was strongly impaired (0.6% and 14% of the wild type). In a double mutant in which the formation of both covalent bonds was impaired, the enzyme was not even capable of binding FAD, yielding an inactive enzyme. Activity on mono- and oligosaccharides was found in a GOO variant from a different Acremonium strictum strain (Fournani et al., 2011) that displayed activity on xylose, galactose and N-acetylgalactosamine, on top of oligosaccharides. Engineering of this variant also yielded catalysts with higher activity on all substrates. This was realised by targeting positions Tyr300 and Trp351 which were mutated to the respective residues present in other carbohydrate oxidases, such as alanine and phenylalanine (Fournani et al., 2011).

The substrate range of GOO made it a relevant target for enzyme engineering. Use of a point mutant Y300A fused to a carbohydrate binding module made the enzyme maintain its activity at high hydrogen peroxide concentrations, while retaining a wide substrate acceptance range and even increasing the specificity for glucose (Vuog et al., 2016). The fusion of carbohydrate binding modules to GOO has been target of studies to increase the efficiency of the enzyme for various substrates. Fusion variants of these modules to a GOO from Physcomitrella patens, both at the N- and C-terminus, were generated and anchored to residues His111 and Cys172. The enzyme has a 10 times higher catalytic efficiency for cellobiose over lactose. Lactose and cellobiose are the best substrates known so far, with $K_m$ values of 0.6 mM and 4.2 mM, respectively. The crystal structures of the wild type enzyme and an active site mutant were solved. While site-directed mutagenesis of residues surrounding the isoalloxazine ring impaired overall efficiency of the redox reaction, the essential role of a valine in regulating the enzyme reoxidation was shown. This could have important implications in future engineering of this and sequence-related oxidases. Chitoooligosaccharide oxidase is typically active on oligosaccharide substrates containing a GlcNAc at the reducing end and is catalysing the oxidation of position C1 of the sugar ring. Being an oxidase, the reaction again involves the use of molecular oxygen and yields hydrogen peroxide, which is made available to the peroxidases, which are abundant in the fungi which have been found to express this protein. ChitO was first cloned from Fusarium graminearum and was characterised for its substrate acceptance. By testing a wide range of mono- and oligosaccharides, it was discovered that ChitO is most active with GlcNAc and its oligomers (chitobiose, chitotriose and chitotetraose). Relatively low activity was observed for some other mono- and oligosaccharides. This sets this flavoprotein oxidase apart from GOO and its close homologs. Using the relatively high sequence homology with lactose oxidase, a homology model-based interpretation for the specific acceptance of N-acetylated carbohydrates was provided (Heuts et al., 2007a). The structural model predicted a role of a glutamine residue at position 268 in recognizing the N-acetyl moiety of the GlcNAc at the reducing end of the substrate. This prediction was confirmed by mutagenesis experiments. The model also allowed to propose the presence of the same FAD binding strategy used by GOO in ChitO (Heuts et al., 2008). Differently form GOO, the ChitO double mutant in Lactose oxidase is also a VAO-type oxidase (Fig. 9) and shares the FAD binding mode with GOO (42% sequence identity). It was the first enzyme with specificity for oligosaccharides to be characterised (Xu et al., 2001). The enzyme displayed higher relative activity on cellobiose (100%) and maltose (83%) than on glucose (69%) and it displayed general stability over different pH conditions (Kulyk et al., 2001). Applications for in vivo use of LAO have been explored, for example in forming the lactobionic acid product, which is of interest for the food industry. The general safety of the enzyme has been tested under deep characterisation of its stability profile and kinetics feature, and its activity was assessed for lactose, which is converted to lactobionic acid, targeting position C1 at the reducing end of the disaccharide (Nordkvist et al., 2007).

A celloooligosaccharide oxidase was found in the plant pathogen Sarocladium oryzae. The oxidase is active on lactose and on cellooligosaccharides and the $K_m$ for such compounds is much lower than the one measured for glucose and maltose (and their oligosaccharides) (Lee et al., 2006). As a comparison, cellobiose dehydrogenases are also known to be active on lactose and related disaccharides and make use of FAD as a cofactor, while the electron acceptors are in this case ranging from quinones to metal ions (Henriksson et al., 2000).

While most known oligosaccharide oxidases are of fungal origin, also some plant oxidases have been described. One recent example is a cellobiose oxidase from the moss Physcomitrella patens (Toplak et al., 2018). Being sequence-related to GOO, this oxidase harbours also a bicovalently bound FAD cofactor, anchored to residues His111 and Cys172. The enzyme has a 10 times higher catalytic efficiency for cellobiose over lactose. Lactose and cellobiose are the best substrates known so far, with $K_m$ values of 0.6 mM and 4.2 mM, respectively. The crystal structures of the wild type enzyme and an active site mutant were solved. While site-directed mutagenesis of residues surrounding the isoalloxazine ring impaired overall efficiency of the redox reaction, the essential role of a valine in regulating the enzyme reoxidation was shown. This could have important implications in future engineering of this and sequence-related oxidases. Chitoooligosaccharide oxidase is typically active on oligosaccharide substrates containing a GlcNAc at the reducing end and is catalysing the oxidation of position C1 of the sugar ring. Being an oxidase, the reaction again involves the use of molecular oxygen and yields hydrogen peroxide, which is made available to the peroxidases, which are abundant in the fungi which have been found to express this protein. ChitO was first cloned from Fusarium graminearum and was characterised for its substrate acceptance. By testing a wide range of mono- and oligosaccharides, it was discovered that ChitO is most active with GlcNAc and its oligomers (chitobiose, chitotriose and chitotetraose). Relatively low activity was observed for some other mono- and oligosaccharides. This sets this flavoprotein oxidase apart from GOO and its close homologs. Using the relatively high sequence homology with lactose oxidase, a homology model-based interpretation for the specific acceptance of N-acetylated carbohydrates was provided (Heuts et al., 2007a). The structural model predicted a role of a glutamine residue at position 268 in recognizing the N-acetyl moiety of the GlcNAc at the reducing end of the substrate. This prediction was confirmed by mutagenesis experiments. The model also allowed to propose the presence of the same FAD binding strategy used by GOO in ChitO (Heuts et al., 2008). Differently form GOO, the ChitO double mutant in

![Fig. 9. Reaction catalysed by lactose oxidase on lactose.](image-url)
which the histidine and cysteine residues involved in FAD binding were substituted, did not express at all. The single mutants, H94A and C154A, could be expressed but, as expected, they were poorly active and displayed a drastic change in the redox potential of the flavin co-factor. The recently elucidated structure of ChitO (Savino et al., 2020) revealed the predicted double covalent linkage, which is conserved when compared to GOO. Also, the presence of different substituents at position C2 of monosaccharides resembling GlcNAc were tested in vitro and analysed in silico. This revealed that the drug streptozotocin and other GlcNAc analogues with various C2-substitutions are also accepted as substrates. In fact, compounds such as valeryl glucosamine were found to be much better substrates than GlcNAc (Savino et al., 2020). The residues composing a secondary pocket in the active site have been found to be responsible for such a broad substrate range. ChitO has been engineered to expand the substrate range to carbohydrates other than GlcNAc and oligosaccharides thereof (Ferrari et al., 2015). This was achieved by introducing single mutations in the predicted carbohydrate binding pocket. As a result, several variants of ChitO were created with distinct substrate acceptance profiles, including variants that behave as a D-glucosamine oxidase or a cellobiose oxidase (Heuts et al., 2008). A variant of the ChitO enzyme having high specificity for cellobiose was fused to a peroxidase (Colpa et al., 2017). This allows detection of low amounts of cellobiose. In fact, it has been shown that ChitO and the variant active on cellobiose can be used for a facile method for detection of chitinase and cellulase activity, respectively (Ferrari et al., 2014).

The xylooligosaccharide oxidase (XyIO) from Thermothenomycys thermophila (previously named Myceliophthora thermophila) was isolated recently (Ferrari et al., 2016). The structures of native and ligand bound XyIO were solved. Just like gluc- and chitoooligosaccharide oxidases, also XyIO performs oxidation at C1 of the substrate, converting for example xylobiose to xylolionate. This other member of the VAO-like family furtherly expands the repertoire of oligosaccharide converting enzymes of fungal origin which seem to be specialised in accepting only specific kinds of monosaccharides and polymers thereof. As observed for ChitO, long oligosaccharides seem to be preferred substrates with respect to shorter oligosaccharides and to monosaccharides. Such a behaviour is facilitated by the higher number of interactions established between longer substrates and the groove leading to the active site (Fig. 4g).

4.2. Sorbitol, xylitol and alditol oxidases

Oxidases active on acrylic sugars like alditols have been found in bacteria, specifically in different Streptomyces strains. The importance of these enzymes derives from their activity, which can be used in biosensors, and from their products, used as building blocks for chemical synthesis. Among these enzymes, sorbitol oxidase (SO) was the first one to be identified (Hiraga et al., 1998) thanks to the high homology (25%) with L-gulonolactone oxidase. A covalently bound FAD is present in SO, but differently from the previously described oligosaccharide oxidases, only the histidyl-FAD linkage is present in these enzymes. Such binding strategy is shared with other alditol oxidases that are described below. Sorbitol oxidase catalyses the C1 oxidation of sorbitol to α-glucose and of xylitol to D-xyllose. The enzyme displays similar substrate specificities with Km values of 0.35 mM and 0.26 mM for sorbitol and xylitol respectively (Hiraga et al., 1997). α-mannititol, D-arabinitol and D-galactitol are also substrates of SO but show much lower activity. The measured melting temperature (Tm) of the enzyme is 55 °C and the pH range is very broad, spanning from pH 5.5 to 10.5. SO, like other alditol oxidases, is a monomeric enzyme. Xylitol oxidase (XO) shares high sequence homology with SO (51% sequence identity) and was isolated from a Streptomyces coming from a hot spring sample (Yamashita et al., 2000). XO converts xylitol to D-xyllose producing hydrogen peroxide (Fig. 10). The pH range for activity of XO is narrower than SO (pH 7.5–10), but the Tm is higher (65 °C), reflecting the thermotolerance of the source organism. The specificity of XO for xylitol is much higher than for sorbitol, going from a Km value of 0.6 mM to 3.5 mM.

Another bacterial alditol oxidase (AldO) was described a few years after the first isolation of xylitol oxidase. This oxidase displayed similar properties such as the higher specificity for xylitol rather than sorbitol (Heuts et al., 2007b). The covalent binding of FAD to His46 was demonstrated by site-directed mutagenesis experiments, and the kinetic mechanism of AldO was studied in detail. The crystal structure of AldO was thereafter solved, revealing most of the predicted features of the enzyme and providing insights in how the reduced flavin cofactor may react with molecular oxygen (Forneris et al., 2008). Due to the high resolution of the AldO crystal structure, it was also used as model to perform MD simulations in order to elucidate how such an oxidase captures and reacts with molecular oxygen. This demonstrated that AldO facilitates and guides oxygen transfer from the surface of the protein to the site where it should react with the reduced flavin cofactor (Baron et al., 2009). Another robust AldO was identified from the thermophilic Acidothermus cellulolyticus, yielding an enzyme with a Tm of 84 °C (Winter et al., 2012) and a substrate profile superimposable to AldO.

Given the preference of AldO for xylitol, engineering was performed on the enzyme to enhance its acceptance of glyceral as a substrate. This would allow to add value to a compound, glycerol, which is produced in huge volumes yearly as a by-product of bio-based fuel production (Gerstenbruch et al., 2012). Using directed evolution, a quadruple mutant was obtained with 2.4 fold higher activity on glyceral with respect to the wild type enzyme, producing the expected glyceric acid product. Further engineering addressed the secretion of the xylitol oxidase from Streptomyces coelicolor (Scheele et al., 2013), which was achieved by expressing the enzyme coupled to a TorA N-terminal sequence and using the host Corynebacterium glutamicum. In this way, the twin-arginine translocation pathway was exploited to secrete a carbohydrate oxidase which is typically a cytosolic enzyme.

Mannitol oxidase activity has been found in the digestive tract of gastropods, but little information is available on the characteristics of such enzymes (Lobo-da-Cunha et al., 2018).

4.3. Aldonolactones oxidases

A group of oxidases present in animals, plants, fungi and yeast can be defined by their common role: production of vitamin C or its analogues. While all these enzymes are carbohydrate oxidases, they are unique in the type of carbohydrates they oxidize: aldonoactones. Dependent on the organism, different molecules take the role of vitamin C, and for this reason, different carbohydrate substrates are used as precursor (Abboobucker and Lorence, 2016). Mammals generally perform their own biosynthesis of ascorbic acid (vitamin C), but curiously this ability has been lost in humans, some primates, bats and guinea pigs. Other animals perform ascorbic acid synthesis starting from UDP-glucuronic acid and following many enzyme-catalysed reactions, the last one of which is the oxidation of L-gulonolactone. L-gulonolactone oxidase (GULO) was isolated at the end of the '80s from Rattus norvegicus (Koshizaka et al., 1988) but its role had been known for decades before, and the presence of a covalent histidyl linkage with FAD had been reported (Kenney et al., 1976). Sequence analysis of GULO clearly indicates that it is member of the VAO flavoprotein family which is in line with the covalently tethered flavin cofactor. Most of the literature on this topic focuses on the inactivation of the genes allowing ascorbate biosynthesis in humans (human have lost a functional GULO-encoding gene) and no structural characterisation is available for the enzyme. A possible reason for the lack of biochemical data on GULO is the fact that it is membrane bound and therefore difficult to express and isolate. Of note is that in this case the reaction catalysed by the enzyme is directed towards the C–C bond between position C2 and C3, where a double bond is introduced (Fig. 11).
Plants use a different precursor, GDP-L-galactose, to produce ascorbate. The difference with UDP-glucuronic acid is in the stereochemistry at C3. The respective L-galactonolactone oxidizing enzyme is not an oxidase but a dehydrogenase, L-galactonolactonate dehydrogenase (GALDH) (Östergaard et al., 1997). It is sequence related to GULO. GALDH is member of the VAO flavoprotein family. Yet, its FAD cofactor is not covalently attached. By a careful sequence analysis of GALDH and sequence related oxidases, a residue was identified that upon replacement (Ala113 to Gly) allowed the enzyme to use oxygen as electron acceptor (Leferink et al., 2009). Thus, GALDH was essentially engineered into a L-galactonolactone oxidase by a single mutation. In fact, the targeted residue is in line with the MD analysis of AldO, engineered into a L-galactonolactone oxidase by a single mutation. In fact, the targeted residue is in line with the MD analysis of AldO, vide supra, which predicted an oxygen access gate keeper role of the respective residue.

In yeast, enzymes reported as FAD-dependent L-galactonolactone oxidases have been found (Nishikimi et al., 1978; Bleeg and Christensen, 1982). The most known oxidase responsible for erythroascorbic acid production in Saccharomyces cerevisiae is arabinonolactone oxidase, a FAD-containing enzyme with the conserved FAD-histidyl linkage (Huh et al., 1998). The parasite Leishmania donovani also expresses an arabinonolactone oxidase but no covalent flavin-protein bond is present and C-terminal residues are essential for FAD binding (Biyani and Madhubala, 2011).

In fungi, the strategy to synthesise erythorbic acid, a stereoisomer of ascorbic acid, is the oxidation of gluconolactone (Salusjärvi et al., 2004). While other enzymes acting on aldonolactones all seem to be monomers, the fungal oxidase has an homodimeric arrangement and it is highly glycosylated, as most secreted fungal enzymes. The residue His67 harbours the covalent linkage to the FAD cofactor.

4.4. Hexose oxidase, plant carbohydrate oxidase and nectarins

A few decades ago, a flavin-containing, highly glycosylated hexose oxidase (HOX) was purified from different red algae (Groen et al., 2004). The substrate scope of this enzyme is quite large, showing similar $K_m$ values for monosaccharides such as glucose and galactose and disaccharides such as lactose, cellobiose and maltoose. The oxidation occurs on the C1 of the sugar molecules. Antimicrobial activity in algae and higher plants has been hypothesised for these oxidases through hydrogen peroxide production (Custers et al., 2004). Other HOX enzymes were found in red algae and their antibacterial activity tested via antibiogram against different bacterial species (Ogasawara et al., 2010). Even in this case similar activities were found for the mentioned substrates. The high glycosylation state of HOX and the presence of a cleavable signal peptide was reported for multiple enzymes and these data suggest an extracellular localisation of HOX. This would be compatible with the antibacterial activity of HOX, or alternatively as a tool to compete with other algae species. Harsh treatment of HOX (Groen et al., 1997) still yielded a FAD-bound enzyme, which suggested a covalently bound flavin cofactor. Upon sequence inspection, the most likely candidates for covalent linkage are a conserved histidine residue and a conserved cysteine residue. While the sequence of HOX shows clear relationship with other VAO-type carbohydrate oxidases, such as GOO, no structure is available for this enzyme.

Carbohydrate oxidases from plants have also been reported with activities similar to the algal HOX (Custers et al., 2004). The sequence homology of these enzymes to other VAO-like members allows to classify them as FAD-containing enzymes although the sunflower and lettuce oxidases were purified without detectable amounts of FAD. From sequence analysis, just like for HOX, a double covalent linkage is predicted to be present between the protein and the isoalloxazine ring of FAD. A possible role of the plant carbohydrate oxidases may lie in pathogen infection response.

Secreted plant proteins from the tobacco sexual organs were identified in the nectar, a solution containing carbohydrates and used to attract pollinators. These proteins were named nectarins and one of them, Nectarin 5 (NEC5), was identified as a flavoprotein (Carter and Thornburg, 2004b). NEC5 is an enzyme present in nectar in three isoforms having a different apparent size on SDS-PAGE. This is caused by different levels of glycosylation at multiple sites on the protein surface. From sequence analysis nectarin 5 can be recognised as a VAO-type flavoprotein. Covalent binding of FAD to the protein was experimentally confirmed and the covalent binding site was found to be a histidine in position 104. Based on the homology with HOX, the oxidase activity of NEC5 was tested for glucose, galactose and mannose. Only glucose was found to be converted to gluconic acid by NEC5. This activity allows to speculate that the role of the enzyme is to generate hydrogen peroxide using the high carbohydrate concentration in nectar (Carter and Thornburg, 2004b). This activity would greatly contribute to the antimicrobial defences of the plant sex organs. The involvement of nectarins in response to pathogens was later confirmed by the finding that nectarin 4, upon interaction with fungal enzymes, triggers NEC5 activity (Harper et al., 2010).

The recently identified structure of uralonic acid oxidase from Citrus sinensis (Wei et al., 2020) adds another biocatalyst to the portfolio of available carbohydrate oxidases. The protein is a member of the VAO family, and its sequence displays the histidine and cysteine residues required for bicovalent binding of the FAD cofactor. The presence of FAD was confirmed via spectroscopy on the purified enzyme, recombinantly produced in yeast.

Fig. 10. Reaction catalysed by xylitol oxidase on xylitol.

Fig. 11. Reaction catalysed by gluconolactone oxidase on L-gulonolactone.
5. Applications of carbohydrate oxidases

5.1. Carbohydrate oxidases as biocatalysts

The use of enzymes in industrial processes for chemical synthesis is often attractive due to the unique properties of biocatalysts, such as their high chemo-, regio- and enantioselectivity and/or the mild conditions required. Carbohydrate oxidases are a good example of biocatalysts that can be used to perform the synthesis of specific products by exploiting their high substrate specificity and exquisite regioselectivity. The highly selective oxidation reactions with carbohydrates are typically extremely difficult to be done using chemical means. Furthermore, the reactions only require mild conditions (and cheap and mild) molecular oxygen as oxidant. The relative ease of expression of most carbohydrate oxidases in complex with their native cofactor makes it easy to adopt them in biocatalytic approaches. The production of hydrogen peroxide on large scale is not an issue. By using catalase in combination with an oxidase, the hydrogen peroxide is recycled into water and molecular oxygen. The efforts made in stabilising the robustness of carbohydrate oxidases and the development of immobilisation strategies is of paramount importance in extending the half-life or reusability of the biocatalysts.

Competing enzymes of carbohydrate oxidases are carbohydrate dehydrogenases, which usually rely on other forms of cofactors such as NAD(P) or cytochromes. Also, these enzymes have been widely studied and received much attention and underwent engineering for developing processes to yield aldehydes, lactones and ketone forms of substrate sugars. Carbohydrate dehydrogenases have their own structural features and substrate acceptance range which can be partially superposed to those of oxidases, as in the recent case of a sorbitol dehydrogenase, which is also capable of converting galactitol to the rare sugar tagatose (Köhlmier et al., 2019). A clear disadvantage of dehydrogenases is the need for cofactor regeneration and often results in a fermentative approach in which cellular metabolism take care of the regeneration.

With the steadily growing number of available carbohydrate oxidas, new applications are being developed. A recent example is the application of carbohydrate oxidases coupled to transaminases in a cascade reaction (Aumala et al., 2019). This leads to the regioselective introduction of amino groups in defined carbohydrate, allowing a one-pot functionalisation of biomass-derived material. Many other applications that involve newly discovered carbohydrate oxidases will surely be developed, but may depend on the ease and costs at which these enzymes can be acquired.

Even for the well-established fungal glucose oxidase many new applications have been reported in the last years. For example, GOX has been used to deplete glucose during synthesis of fructo-oligosaccharides, avoiding the inhibitory activity that glucose has on the transglycosylating enzyme used in the reaction (Valdivieso-Ugarte et al., 2006). GOX activity has also been coupled to fructofuranosidase by co-immobilisation to produce lactosucrose, an important prebiotic that helps the intestinal microflora fitness (Long et al., 2019). GOX has been used already for years to produce gluconic acid (Godjevargova et al., 2004) added to food as an antioxidant. Recently, the biosynthesis of this compound by GOX was boosted by using the enzyme variant from a strain of Aureobasidium pullulans (Zhang et al., 2019). Co-immobilisation of glucose oxidase and catalase was also used to obtain efficient production of gluconic acid from lignocellulosic material with high yield (Han et al., 2018). An in-depth review on the applications of fungal glucose oxidases covers most of the uses of the enzymes predating the ones we reported here (Dubeby et al., 2017). GOX is also applied in D-glucosaminic acid synthesis which can be used for biomedical applications (Pezzotti et al., 2005; Zhang et al., 2010).

Pyranose oxidase is widely employed because of its possibility of oxidising alternative positions of the sugar moiety when compared with GOX. Oxidation of α-glucose at C2 yields D-glucosone (Giffhorn et al., 2000), which is a precursor for the synthesis of other monosaccharides.

The general efficiency of the reactions performed by this enzyme is higher than GOX and engineering of POX delivered mutants with novel reactivity for L-sorbose, and increased conversion to ketones (e.g. 5-keto-Δ-l-fructose), relevant building blocks for other chemicals, such as iminosugars (Bastian et al., 2005). Iminosugars are used as inhibitors for medicinal and research purposes (Schneider et al., 2012). The molecular basis for the regioselectivity of POX for position C3 has been thoughtfully elucidated (Kujawa et al., 2006) and this unusual property can be exploited for the synthesis of rare sugars (Freimund et al., 1998). Furthermore, on this line, the enzyme has been recently applied to synthesise ribulose starting from arabinose in a one-pot enzymatic cascade (Chuabo et al., 2019). On top of the oxidase activity, quinones can also be used as electron acceptors, something which has been used in experiments to regulate lignin depolymerisation (Ai et al., 2014).

Alditol oxidase was recently shown to be useful for the synthesis of rare sugars. Through cascade catalysis (Chen et al., 2020) or one-pot systems (Li et al., 2020), the synthesis of D-sorbose and D-psicose starting form glycerol was demonstrated.

Galactose oxidase can also be used thanks to its wide substrate range for the synthesis of other compounds such as l-fructose (Franke et al., 2003), 2,5-diformylfuran, and 2,5-furan dicarbonylic acid (Cajnko et al., 2020). Recently GAO also showed the potential to produce amino carbohydrates in combination with transaminases (Aumala et al., 2019) and to convert alcohols to nitriles, passing through the aldehyde intermediate and using an ammonium buffer as source of nitrogen (Vilim et al., 2018).

5.2. Other biotechnological uses of carbohydrate oxidases

Carbohydrate oxidases have found many applications in fields other than the biosynthesis of rare sugars and related compounds. The reaction mechanism of these enzymes makes them also ideal candidates for biosensors, taking advantage of the consumption of oxygen or the formation of hydrogen peroxide to monitor the specific reactivity on substrates, such as medically relevant marker molecules. For this reason, biosensors have been developed in which carbohydrate oxidas constitute the sensing element, generating a signal that can be converted into an electric one (Jaffari and Turner, 1995).

An extremely high number of references and patent is available concerning the use of glucose oxidase in biosensors (Wilson and Turner, 1992; Bankar et al., 2009). The market value for biosensors has been estimated to be around 5 billion dollars back in 2005 (Newman and Turner, 2005), with GOX occupying most of it. This shows that GOX is playing a major role in the development and application of GOX in sensor devices. Among the most popular applications in which GOX is mentioned, is the monitoring of glucose in blood of diabetes-affected individuals. The same principle used in this kind of technology can be applied to continuous monitoring systems (Wong et al., 2008) which can be used for blood samples but also for determining sugar content in drinks (Chudobova et al., 1996). In this field, oxidases share the pri-mate with dehydrogenases (Freckmann et al., 2012), which have also been studied and enhanced to be used as biosensors (Arango Gutierrez et al., 2013; Baik et al., 2003). An important aspect of biosensor development is the stabilisation of the oxidase involved (Mano, 2019; Harris et al., 2013) which can be achieved by directed evolution or by identifying elements of instability such as high hydrogen peroxide concentrations. An equally important contribution in the development of biosensors comes from material science, which provides new solutions in the form of nanomaterials to support the biocatalyst and allow the generation of the electric signal (Ge et al., 2019; Yu et al., 2019). Also, enzyme engineering can be of use. Recently, the fusion of carbohydrate oxidases to peroxidases was shown to be possible, allowing a sensitive biosensing system for specific carbohydrates which relies only on one biocatalyst (Colpa et al., 2017).

Huge efforts have been made in using artificial acceptors different...
from oxygen, avoiding accumulation of hydrogen peroxide while generating a specific signal for the reported molecule, for example a dye (Kim et al., 2010). Similarly, attempts have been made in engineering oxidases to ignore molecular oxygen as an acceptor, in this way making the signal for biosensing system insensitive for the oxygen dissolved in solution (Mano, 2019; Ferri et al., 2009; Kim et al., 2012). One of the most recent attempts to further improve the use of GOX in biosensing focuses on the “third-generation” sensor principle. This aims at exploiting direct electron transfer from glucose oxidation directly to an electrode, without intermediates. For such approach, lysines have been introduced on the surface of GOX, allowing a chemical modification with a redox mediator, re-oxidising the FAD cofactor upon reduction by glucose (Suzuki et al., 2020).

GOX is being studied also for multiple other applications such as enzymatic biofuel cells development (Alcaraz et al., 2016), bleaching of textiles and as a food additive. The latter two applications exploit the hydrogen peroxide production of GOX. Textiles can be bleached using the hydrogen peroxide generated by GOX (Tzanov et al., 2002), and it is even possible to use a coupled reaction with a peroxide-dependent peroxogenase (Opwis et al., 2008). When used as a food additive, the scopes of GOX are manifold: it removes oxygen and glucose, prolonging food shelf life, and it generates hydrogen peroxide, which acts as bactericide. Hydrogen peroxide can be converted by catalase to water and for this reason the two enzymes are often used together (Crueger and Crueger, 1990). Indirectly, GOX is relevant to food industry also as a source of gluconic acid (Wong et al., 2008; Crueger and Crueger, 1990). The potential of GOX is spread also in different directions, such as groundwater remediation from chlorinated compound (Huang et al., 2020) and it is beyond the scope of this review to explore all the exotic applications of this versatile enzyme.

The use of oxidases in biosensors is not exclusive of GOX. Galactose oxidase has been employed for samples such as milk, to detect production of hydrogen peroxide, using different strategies of immobilisation (Kanyong et al., 2017). Pyranose oxidase similarly found many applications in biosensors and also in biofuel cells. Differently for glucose oxidase, POX can be used to detect more sugars (Abera et al., 2020) such as xylose, galactose, mannose, and maltose, depending on the origin of the POX employed. Taking maltose as example, its detection is particularly relevant in beer manufacture, and for this different methods have been used to monitor and quantify it. A bi-enzymatic system was developed to assess maltose using POX in combination with α-glucosidase (Odaci et al., 2010). POX is nowadays often regarded as a preferable alternative to GOX in the construction of biosensors, thanks to the absence of glycosylation sites and the higher catalytic efficiency. The use of POX in biofuel cells, which produce energy on a small scale exploiting the enzymatic reaction of the oxidase, is dependent on the use of electron acceptors different from molecular oxygen, such as quinones or ferrocenium ions (Abrera et al., 2020, Spadiut et al., 2009a, 2009b) and saturation mutagenesis has been used as an approach to construct improved variants making use of such acceptors. Immobilisation is an important aspect of this application of the oxidase and it has been addressed in various ways (Kim et al., 2017). Other uses of POXs are extended to textile bleaching (Pazarioglu et al., 2012) and to improvement of dough stability and bread quality through formation of cross linking between gluten proteins, caused by hydrogen peroxide (Decamps et al., 2013).

The use of lactose oxidase in food industry has been directed towards pathogen inhibition in dairy products, reducing spoilage caused by pathogens such as Escherichia coli, Salmonella enterica, and Staphylococcus aureus (Lara-Aguilar and D Alcaine, 2019). This effect is caused by an increased concentration of hydrogen peroxide caused by the activity of LAO, resulting in the activation of a bactericidal lacto-peroxidase system.

Hexose oxidase is also used in food industry, in this case to control the extent of Maillard reaction occurring in baked products (Søe and Petersen, 2005). HOX is used to oxidise the reducing end of sugars which would react with an amino acid in a Maillard reaction. This is meant to avoid excessive browning of some food products for which this feature is not desired. Maillard reaction control is also the target of applied research on fructosyl amino acid oxidase (Troise et al., 2016) which can be used to drastically reduce the glycation of amino acids (Amadori products) and consequently the occurrence of the Maillard reaction. Fructosyl amino acid oxidase and fructosyl peptide oxidase underwent immobilisation studies (Chen et al., 2019; Chen et al., 2018) with the purpose of maintaining the enzyme activity while being used for the detection of glycated haemoglobin, an indicator of diabetes. Chitooligosaccharide oxidase has demonstrated potential for application in biosensors detecting chitinase and cellulase activities, as an alternative to established but more laborious chemical methods (Ferrari et al., 2014).

6. Conclusions

Carbohydrate oxidases form a versatile group of enzymes, with each member displaying specific activity towards one or a small group of carbohydrates. Research in the last decades has shown that new carbohydrate oxidases are continuously discovered (Table 1). Many crystal structures have been solved, which help in understanding the unique catalytic properties of the various carbohydrate oxidases including their interactions with their substrates. Such structural information is of particular help when redesign of a carbohydrate oxidase is required through enzyme engineering. The applicability of carbohydrate oxidases industrial scale for synthetic purposes has been demonstrated to some extent, and the high specificity and unique oxidative properties of these enzymes also allowed their application as biosensors for biomedical use and for food preservation. To summarise, the research on carbohydrate oxidases has yielded important results in the last three decades, for a large part due to the growing number of known and characterised carbohydrate oxidases.

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