ENGINEERING GALACTOSE OXIDASE TO INCREASE EXPRESSION LEVEL IN *E. coli*, ENHANCE THERMOSTABILITY AND INTRODUCE NOVEL ACTIVITIES

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Abstract

For the first time, we functionally expressed the fungal glycosylated copper-containing enzyme, galactose oxidase, in *Escherichia coli*. The generation of fully functional GOase confirms that the formation of the unusual thioether bond is an auto-catalytic process involving a self-processing mechanism. This process does not rely on the pro-peptide of native GOase and is not dependent on the auto-cleavage of the precursor peptide during maturation, a question which can not be illustrated with other expression systems. The kinetic parameters of recombinant GOase and native GOase are comparable, but the former is less stable than the latter, revealing that the small contents of carbohydrate in native GOase confer a more rigid structure, but not a beneficial effect on catalytic efficiency to the enzyme. We applied directed evolution to generate GOase variants with improved thermostability, increased catalytic efficiency, and enhanced expression level in *Escherichia coli*. The final mutants are comparable to fungal GOase in their thermostability, long-term stability and radical stability. We also applied saturation mutagenesis to endow the protein with a novel D-glucose 6-oxidase activity, an activity that has never been reported in nature or laboratory. The resultant mutant M-RQW, with three amino acid substitutions (R330K, Q406T/S and W290H), shows low but significant activity in the selective oxidation of D-glucose's 6-hydroxyl group and has decreased activity towards its native substrate, D-galactose. None of the mutations can be obtained by single nucleotide substitutions. The mutation on W290, a residue proposed to stabilize the radical of GOase, supports the conclusion that W290 restricts substrates

from entering the active center of GOase. Moreover, kinetic characterization of the mutant indicates that W290 might facilitate access of D-galactose to the active center. It is also found that radical stability is not only affected by reduction potential, but also structural factors. The synergistic interactions between the R330K and Q406S/T substitutions improve D-glucose accessibility to the active center. Mutant M-RQW can also accept aliphatic secondary alcohols to generate corresponding ketones. This activity, compared with the bioinorganic mimics of GOase, illustrates the flexible, yet robust, active center configuration of GOase.

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Chapter I Introduction

1. Galactose Oxidase

1.1. General Properties

Galactose oxidase is a glycosylated protein secreted by fungus *Fusarium spp.*, previously identified as *Dactylium dendroides* and *Polyporus circinatus* (Amaral et al., 1963; Avidad et al., 1962; Cooper et al., 1959). Mature galactose oxidase (GOase, oxidoreductase: galactose 6-oxidase, EC 1.1.3.9) contains 639 amino acids and one cupric ion, and has a molecular weight of 68 kDa. Carbohydrate constitutes approximately 1% of intercellular GOase and 7.7% of intracellular GOase (Mendonca and Zancan, 1987). Before GOase is secreted from fungal cells, a peptide containing 41 amino acids is removed. This peptide contains a signal sequence necessary for the transport of GOase out of cells and a precursor sequence that was proposed to facilitate folding of GOase (McPherson et al., 1993).

Native (fungal) GOase exhibits unusual resistance to denaturation and reduction. It is still active in 6 M urea and its thiol groups are difficult to titrate with 5,5'-dithio-bis(2-nitrobenzoic acid). These findings indicate an inherent conformational stability and inaccessible cystine and/or cystine groups of GOase (Kosman et al., 1974). The unusually high isoelectric point of GOase (pI=12) accounts for its high affinity to glass and macromolecules, and its inability to be removed without a dilute acid wash (10% HNO_3) (Kosman et al., 1974).

GOase catalyzes the oxidation of the 6-hydroxyl group of D-galactose into a corresponding aldehyde, with oxygen being reduced to hydrogen peroxide. When the hydrogen peroxide accumulates during catalysis, GOase is irreversibly inactivated. However, when there are no substrates present, GOase is stable to hydrogen peroxide (Hamilton et al., 1977). In higher concentrations, oxygen retards the inactivation power of hydrogen peroxide. Some oxidizing reagents, such as ferricyanide and EDTA complex of Mn(III), significantly increase the rate of GOase catalysis (Cleveland et al., 1975; Hamilton et al., 1977). Peroxidase, which is commonly used to assay GOase activities, also activates GOase. The oxidation of GOase by the intermediate radical with high redox potential produced in the peroxidase reaction was proposed to explain the activation of GOase by peroxidase (Hamilton et al., 1977).

1.2. Three-dimensional Structure of GOase

The crystallographic structure of GOase at pH 4.5 reveals a three-domain structure dominated throughout by β-sheets, and domain I uniquely contains a single, short α-helix (Figure 1.1) (Ito et al., 1991; Ito et al., 1994). The first domain (residues 1-155) has a jelly-roll motif with a five-stranded antiparallel β-sheet facing a three-stranded antiparallel β-sheet. This domain does not contain any active center residues and is not expected to be directly involved in the catalytic process. However, domain I is resistant to proteolytic attack and is believed to facilitate the folding of GOase since the deletion of domain I led to an inactive form (McPherson et al., 1993). Domain II (residues 156-532) has a

pseudo sevenfold symmetry, where seven β-sheets surround a cavity and each β-sheet contains four antiparallel β-strands. Cupric ion is close to the central axis, with three (Y495, H496, Y272) of the four ligands at the solvent-accessible bottom of domain II. Domain III (residues 533-639) contains seven β-strands surrounding a hydrophobic core, stacking above the top of the second domain. One antiparallel β-ribbon pierces the middle of the second domain along the pseudo sevenfold axis to provide a ligand (His 581) for the copper.

The active center of GOase contains four residues: Y495, H496, Y272 and H581 (Figure 1.1). These four ligands and one water molecule, or acetate ion, bind to the cupric ion with square pyramidal coordination. An unusual thioether bond between ligand Y272 and residue C228 is found, and one W290 residue stacks above the top of the Tyr-Cys bridge. This structure supports the radical reaction mechanism (Whittaker and Whittaker, 1988), where the Tyr-Cys cluster is the presumed position of the radical. Formation of the thioether bond greatly reduces the redox potential of tyrosine from 900 mV (free tyrosine) to 410 mV – the lowest redox potential of tyrosine in nature (Borman et al., 1998). Also revealed in the crystal structure, spectroscopic results established that substrates bind to the cupric ion at an equatorial position to replace water or acetate (Knowles et al., 1995).

A calculation of the water-accessible surface of GOase active center reveals a pocket at the copper site which is structurally complementary to the chair form of

D-galactose (Ito et al., 1994). In addition, this substrate binding model shows that R330 and Q406 form hydrogen bonds with O4 and O3 of D-galactose. The backbone of D-galactose has hydrophobic interactions with the side chains of two phenylalanine residues (F464 and F194) of the pocket wall. This model also indicates that D-glucose, a 4-epimer of D-galactose not accepted by GOase as a substrate, would break the hydrogen bond with R330 and cause serious steric hindrance with Y495, a copper ligand.

1.3. Reaction Mechanism

The reaction mechanism of GOase has attracted tremendous interest since GOase seems to employ a single cupric ion, which usually mediates a one-electron transfer process, to catalyze a two-electron transfer reaction. Since purified GOase can be oxidized to a more active form, a mechanism involving the conversion between Cu(III) and Cu(I) was proposed (Hamilton et al., 1977). However, XANES (X-ray absorption near-edge structure) spectra for GOase revealed no presence of Cu(III) (Clark et al., 1990). A PQQ cofactor mechanism was proposed, but not supported by any evidence (van der Meer et al., 1989). Whittaker and coworkers were the first to prepare the stable and homogeneous forms of GOase and proposed that radicals are involved in the catalytic reaction (Figure 1.2) (Whittaker and Whittaker, 1988). A series of elegant experiments by Whittaker revealed the copper oxidation states of GOase before and after oxidation (Clark et al., 1990), the presence of an EPR-active tyrosyl radical in apo-GOase, and an EPR-silent tyrosyl radical in holo-GOase (Whittaker et al.,

1989; Whittaker and Whittaker, 1990; Whittaker and Whittaker, 1993). The radical mechanism gained further support from crystallographic data, which showed the presence of a thioether bond between Cys228 and Tyr272 (Ito et al., 1991), and site-directed mutagenesis studies (Baron et al., 1994).

The crystal structure of GOase shows that acetate ion (pH 4.5), or water (pH 7.0), occupies one equatorial position of the square pyramidal coordination of the copper ligands. The spectroscopic results, furthermore, demonstrated that substrates bind to copper in an equatorial position since the substrate dihydroxyacetone replaces the water at pH 7.0 (Knowles et al., 1995).

The first step in the proposed radical reaction mechanism is the transfer of a proton, from the acidified hydroxyl of the coordinated alcohols, to a copper ligand, Y495. The ligand Y495 is around 2.7 angstroms from the copper and forms a relatively weak bond. It functions as a general base in the catalytic reaction mechanism, a functionality supported by an azide binding experiment (Whittaker and Whittaker, 1993) and a site-directed mutagenesis study (see below) (Reynolds et al., 1995). After the deprotonation of the hydroxyl group and dissociation of Y495 from copper, substrates are converted to better reducing agents, alkoxides, and the substrates are activated. A *Pro*-S hydrogen atom is then abstracted from the adjacent methylene group by the Y272 phenoxyl radical, leading to a partially oxidized radical substrate. The Cu(II) is then reduced to Cu(I) with the oxidation of the partially oxidized substrates to

aldehydes. The homolysis of the C-H bond and electron transfer to Cu(II) can be achieved through a concerted or a stepwise mechanism (Wachter and Branchaud, 1996). The resulting 3-coordinated Cu(I) intermediates are rapidly oxidized by O_2 to form H_2O_2 as the final product. This ping-pong radical mechanism is supported by various computational (Himo et al., 2000; Wachter and Branchaud, 1996) and experimental studies (Branchaud et al., 1993; Turner and Branchaud, 1999). Furthermore, the significant kinetic isotope effect of substrate oxidation (k_H/k_D=21) establishes that hydrogen abstraction of the hydroxymethyl group is the rate-limiting step (Whittaker et al., 1998).

However, the role of the thioether bond in the catalytic reaction still remains ambiguous. It is well known that tyrosyl radicals of native GOase can exist in two stable states: an oxidized Cu(II)-Tyr$^{\bullet}$ state and a semireduced Cu(II)-Tyr state. These states can be interconverted by inorganic oxidation and reduction. One method to distinguish these two states is with their UV-Vis absorbance: the reduced state exhibits absorbance at wavelength 450 nm, while the oxidized state shows strong absorbance at wavelengths 450 nm and 800 nm. The absorbance at 450 nm has been assigned to phenolate (Y495)-to-Cu(II) ligand-to-metal charge transfer (LMCT) and $\pi \rightarrow \pi^*$ transitions of Y272, while the absorbance at 800 nm has been proposed to be derived from a ligand-to-ligand (Y495-Y272) charge transfer, Cu(II) d $\rightarrow$ d, and intraradical absorption (Whittaker and Whittaker, 1998). The charge transfer between Y272 and W290 may also contribute to this absorption (Baron et al., 1994). The formation of a thioether

bond decreases the redox potential by 500 mV or more, suggesting that the thioether bond seems to fine tune the redox potential of the tyrosine, augmenting its stability and accessibility for catalysis (Whittaker and Whittaker, 1998). However, further experiments indicate that the unusually low redox potential of the tyrosine radical in GOase is additionally influenced by the presence of copper and ligand Y495 (Wright and Sykes, 2001). A theoretical study shows that the thioether bond formation has very minor effects on the energetics of the reaction, suggesting its roles are structural rather than mechanistic (Himo et al., 2000). However, QM/MM Car-Perrinello simulation results indicate that formation of the thioether bond stabilizes the enzymatic transition state by delocalizing the unpaired spin density over the cross-linked C228-Y272 (Rothlisberger et al., 2000). Further research is required to fully elucidate the role of the thioether bond in GOase.

Biogenesis of the thioether bond in GOase still remains unknown. A mechanism requiring oxygen and cupric ion to remove the precursor peptide as well as to form the thioether bond has been established, but the detailed chemistry of this mechanism is still uncertain (Rogers et al., 2000). The crystal structure of GOase with the precursor peptide indicates that various amino acid residues might be involved in this self-catalyzed process (Firbank et al., 2001). Galactose oxidase joins an increasing number of enzymes that utilize an autocatalytic posttranslational modification to form cofactors, for example, amine oxidase,

whose biogenesis of topaquinone cofactor has been well established as an autocatalytic process (Dooley, 1999).

1.4. Substrate Specificity and Stereoselectivity

Native fungal galactose oxidase has a relatively broad substrate specificity. GOase shows high reactivity with its native substrate D-galactose, having K_m of 67 mM and k_{cat} of 2990 s^{-1} (Baron et al., 1994). D-galactose derivatives, 2-deoxy-galactose, galactosamine, and numerous galactose polysaccharides, are also active with native GOase. Moreover, polysaccharides containing D-galactose at the reducing end are usually more active than D-galactose with GOase, primarily due to a decreased K_m. Guaran, a high molecular weight mannan containing branched terminal galactose units, has a K_m with GOase 1000 times smaller than galactose (Avidad et al., 1962). The small K_m is also observed for D-galactose conjugates with cholesterol-bearing pullulan (Taniguchi et al., 1999) and liposomes with galactose residues of the surface (Ohno and Kitano, 1998). The oxidation of oligosaccharides and polysaccharides by GOase is consistent with the proposed substrate binding model, in which substrates bind to copper at an equatorial position and the long chain of the polysaccharide is exposed to the solvent (Ito et al., 1994). The high affinity of GOase to polysaccharides is probably related to the high basicity of GOase.

GOase is also active towards glycoproteins with terminal or subterminal D-galactose units (Goudsmit et al., 1984). This reactivity has led people to apply

GOase to oxidize glycoproteins, which are then treated by the reducing agent NaB^3H for labeling. Various primary aliphatic alcohols are also substrates of GOase, but with lower activity compared to D-galactose. Dihydroxyacetone was found to be the best substrate of GOase, with a K_m by threefold lower than for D-galactose.

GOase stereoselectively abstracts the *Pro*-S hydrogen atom of the hydroxymethyl group of D-galactose and methyl-β-D-galactopyranoside (Maradufu et al., 1971; Ohrui et al., 1987). GOase also stereoselectively oxidizes glycerol to generate S(-) glyceraldehydes (Klibanov et al., 1982). D-glucose, a C-4 epimer of D-galactose, cannot be oxidized by GOase. In fact, even at the concentrations as high as 1M, D-glucose does not interfere with the D-galactose oxidation reaction (Wachter and Branchaud, 1996). Due to its stereoselectivity, GOase is a valuable catalyst in organic synthesis.

The stereospecificity of GOase is consistent with molecular modeling research (Wachter and Branchaud, 1996). When a *Pro*-S hydrogen is positioned to react with protein radicals, a maximal number of hydrogen bonds and favorable van der Waals interactions are formed. A transition state model of D-glucose binding shows that three of five hydrogen bonds disappear and O4 sterically clashes with a ring carbon of Y495, which was proposed by a molecular docking experiment (Ito et al., 1994). Unfavorable interactions between D-glucose sugar hydroxyl groups (O3, O4 and O5) and the hydrophobic surface consisting of F464, F195,

W290 and Y495 are proposed by the molecular docking experiment and confirmed by the molecular modeling research. The combination of unfavorable interactions and steric clash block D-glucose from accessing the active center of GOase.

1.5. Functional Expression of GOase

Production of native GOase by *Fusarium spp.* NRRL 2903 is an inefficient and time-consuming process. Genetic operations on GOase and various biochemical characterizations require a recombinant expression system to efficiently generate functional GOase. The first functional expression of GOase was accomplished in fungus *Aspergillus nidulans* regulated by its own promoter or an inducible *gla*A (glucoamylase) promoter. In this expression system, a yield as high as 50 mg per liter culture (compared to 5 mg/liter in native fungus) was achieved (Baron et al., 1994). The expressed GOase is essentially identical to native GOase, both in terms of kinetic parameters and spectral properties. All the mutational studies on GOase were performed in this expression system, and the corresponding purified GOase was used for crystallization (Baron et al., 1994; Firbank et al., 2001). The functional expression of GOase in fungi was also reported in strains of *Fusarium venenatum* and *Aspergillus oryzae* (Xu et al., 2000).

Thus far, the best generation of functional GOase is accomplished with the *Pichia pastoris* expression system (Whittaker and Whittaker, 2000). With this system, the yield of GOase is as high as 500 mg per liter culture. This expression

system is a convenient way to generate functional GOase that can be used for numerous applications. An application in organic synthesis will particularly benefit from this efficient GOase expression system, since the application is primarily limited by the availability of the enzyme (Mazur, 1991).

However, functional expression of GOase in *E. coli* is still challenging. It is advantageous to use *E. coli* due to its fast growth rate and well-established, convenient genetic operations. However, expression of GOase in *E. coli* generated an inactive form of GOase, though it represented approximately 3% of the total cell protein (McPherson et al., 1993). The presence of leader sequence, proposed to be important for correct GOase folding in *E. coli*, can encourage a small amount of active GOase to form (McPherson et al., 1993). The successful expression of GOase as a fusion protein with *Lac*Z or glucan, the binding domain of glucosyltransferase-S, in *E. coli* was achieved, and the latter construct was used to control dental plaque bacteria (Lis and Kuramitsu, 1997). The fusion nature of this construct, however, makes it inconvenient for the biochemical characterization of GOase. The functional expression of the entire GOase gene, the mature gene and leader sequence, *gao*A, was accomplished in *E. coli* and used to develop a high-throughput solid-phase screening method to apply directed enzyme evolution to improve total activity of GOase towards guar, a carbohydrate polymer with a non-reducing D-galactose (Delagrave et al., 2001). The presence of precursor peptide in the GOase from this system makes it less attractive for some mechanistic studies.

1.6. Applications of GOase

The unique features of GOase have attracted a great deal of interest regarding the application of this enzyme in various fields. The excellent selectivity of GOase towards D-galactose and its derivatives has been used to develop analytic tools to assay the carbohydrate contents of various mixtures. Biological sensors based on galactose oxidase (Szabo et al., 1996; Tkac et al., 2000; Vrbova et al., 1992) have been developed to monitor the contents of galactose, galactose derivatives, and other sugars (Schumacher et al., 1994) and utilized in food research (Adanyi et al., 1999; Mogele et al., 1993), bioprocess monitoring (Szabo et al., 1996), and clinical analysis (Vrbova et al., 1992). GOase has also been employed as an assay reagent to measure the activities of sialidase (Alon et al., 1991) and endo-type carbohydrases (Lee et al., 1995). A method based on GOase was developed to measure the concentrations of polysaccharides and proteins in two-phase mixtures. This method has shown promise for the quantitative assessment of phases containing carbohydrate polymers as well as in studies of their morphological changes occurring during thermo-mechanical processing (Nordmark and Ziegler, 2000). The application of GOase to oxidize complex biantennary oligosaccharides to introduce fluorophore acceptors enables researchers to use fluorescence energy transfer to study the conformation of each antenna (Stubbs et al., 1996).

As a consequence of its regio-specificity and stereo-specificity, GOase has been used to synthesize unusual sugars and 5-C(hydroxymethyl) hexoses (Mazur and Hiler, 1997; Root et al., 1985), which are normally difficult to synthesize through chemical methods. Through this efficient catalytic process, as much as 10 kg of products can be generated (Mazur, 1991). GOase was also used to prepare sugar-containing polyamines (Liu and Dordick, 1999), and L-glucose, L-galactose and L-xylose (Yadav et al., 2002). Numerous intermediates in organic synthesis have been prepared using GOase (Bulter et al., 2001; Kimura et al., 1996; Lang et al., 1992). Since the coproduct hydrogen peroxide inactivates GOase, catalase is generally used to efficiently catalyze the decomposition of hydrogen peroxide. For the anaerobic regeneration of GOase and prevention of the production of hydrogen peroxide, an electrochemical method was developed, which may facilitate the large scale application of GOase in organic synthesis (Petersen and Steckhan, 1999).

GOase has been a valuable tool for medical research. Sham's test, a simple method using GOase to detect sugar moieties from rectal mucus, is regarded as a complementary test for immunological fecal occult blood test (FOBT) in the screening of colorectal carcinomas (Zhou et al., 1993). GOase was used to abolish the transfection ability of galactosylated histone H1 and asialo-orosomucoid in gene therapy research (Chen et al., 1994). GOase has been used as a tool to assay the disaccharide tumor marker Gal-GalNac (D-galactose –β[1-3]-N-acetyl-D-galactosamine) generated by patients with colorectal cancer,

and this assay method has been a cost-effective screening test of the patients (Said et al., 1999). The expression of this marker in carcinomatous breast, ovary, pancreas, stomach, and endometrium has expanded the use of this simple screening method for many types of cancers (Shamsuddin et al., 1995). GOase has been used in the detection of mucins for pathological diagnosis (Ota et al., 1995), early detection and prognosis of human colorectal adenocarcinoma (Carter et al., 1997), assessment of invasion depth of gastric carcinomas by endosonography (ES) (Makamura et al., 1997), and evaluation of the Billroth II technique and Rouxen-Y procedure for reconstruction of gastric stump (Kobayasi et al., 1994). GOase–glucan binding domain fusion proteins have been used to inhibit dental plaque bacteria (Lis and Kuramitsu, 1997).

GOase has been used to treat oligosaccharides (Martin et al., 1998) and manufacture sweeteners and flavorants for the food industry, and produce paper strength additives for the paper industry (Xu et al., 2000) (Delagrave et al., 2001). Galactose oxidase can be used to oxidize the soluble polysaccharides of membrane-bound glycoproteins containing terminal or non-reducing galactose residues, which has been a highly successful way to radiolabel these compounds (Light, 1986). Reports also indicated that galactose oxidase is employed in hair coloring and waving (Tsujino et al., 1991).

1.7. GOase Engineering

Early efforts to engineer GOase focused on illustrating its reaction mechanism. Although a radical reaction mechanism was proposed (Whittaker and Whittaker, 1988), the amino acid residues involved were not known until the three-dimensional structure of GOase was solved (Ito et al., 1994). To verify the roles of these residues (H495, H581, Y495, Y272, C228 and W290) and the radical positions (Y272, C228), a series of site-directed mutagenesis experiments was conducted. The thioether bond between C228 and Y272 was removed by the substitution C228 with G228 (Baron et al., 1994). The absence of the thioether bond in mutant C228G led to a fully extended galactose oxidase polypeptide, which can be distinguished from native GOase by its mobility during SDS-PAGE. The formation of the thioether bond in native GOase generates a peptide loop leading to a less extended conformation of the polypeptide chains with a faster migration rate during SDS-PAGE. In the studies of its biogenesis, this method has been used to examine the thioether bond formation (McPherson et al., 1993; Rogers et al., 2000). The catalytic efficiency of mutant C228G is approximately 5000–10,000-fold less than that of native GOase, although its affinity for D-galactose remains unchanged. The three-dimensional structure of C228G is essentially identical to native GOase. The positions of the copper and its ligands remain unchanged, though slight changes of residues W290, F194 and F227 are observed. The diminished copper content (0.25 mol copper/mol enzyme vs. 1 mol copper/mol enzyme in native enzyme) in this mutant indicates that C228 contributes to copper binding. Mutation W290H was introduced into fungally

expressed recombinant GOase to study the role of W290 in the radical by retaining a heterocyclic ring (Baron et al., 1994; McPherson et al., 1993; Rogers et al., 1998). The mutant W290H has 1000–2000-fold less activity than native GOase. Its three-dimensional structure only reveals local changes around the mutation site, and the structure of the copper ligands has no significant differences from the native GOase structure. The increased redox potential (730 mV vs. 410 mV for native GOase) of the mutant W290H indicates the involvement of W290 in maintaining a low redox potential and stabilizing the radical. In the mutant W290H, the increased accessibility of the active site to various buffers suggests the role of W290 in impeding substrates from entering the active site of GOase. Compared to native GOase, both mutants showed dramatic changes in spectral properties. The absorbance at 450 nm in native GOase, which is generated by a charge transfer between Y495 and Cu(II), and $\pi \rightarrow \pi^*$ transitions of Y272 (Whittaker and Whittaker, 1998), shifts to 476 nm, indicating that the stacking of W290 and the C228-Y272 thioether bond influence the $\pi \rightarrow \pi^*$ transitions. The remarkable agreement between the decreased absorbance at 476 nm and the catalytic efficiency of C228G, with its low copper occupancy, suggests C228 influences the radical site in a similar manner as W290.

The role of copper ligand Y495 as a general base was verified by the study of a mutant Y495F (Reynolds et al., 1995; Rogers et al., 1998). This mutant is 1100-fold less active than native GOase, and its three-dimensional structure remains

essentially unchanged except the loss of the tyrosyl hydroxyl group. Since the mutant Y495F is unable to accept protons, this suggests Y495 functions as a general base in the GOase catalytic mechanism.

Random mutagenesis combined with a high-throughput digital imaging screen has also been used to increase the activities of GOase (Delagrave et al., 2001). The best mutant had 16-fold increased total activity than wild-type GOase and a 3-fold decrease of K_m.

2. Saturation Mutagenesis

Saturation random mutagenesis has long been used to investigate protein-DNA interactions and protein functions (Brunet et al., 1993; Clarke et al., 1988; Horwitz and Loeb, 1986; Wells et al., 1985). In this technique, a single amino acid codon is mutated to all other codons encoding the 20 naturally occurring amino acids. Individual clones are then identified by either a genetic selection procedure or a screening method. This technique can be employed to simultaneously mutagenize several codons (combinatorial saturation mutagenesis), either in contiguous blocks or in separate positions, allowing the exploration of all possible combinations and permutations of interesting amino acid residues in order to identify their optimal interactions and geometry for desirable protein properties.

It is believed that nature uses a relatively small number of protein folds to construct numerous proteins with diverse functionalities (Thornton et al., 1999). Enzymes with analogous catalytic functions, but different substrate preferences, are suggested to have evolved from a common structural framework (Perona and Craik, 1997). These enzymes have conserved amino acid residues essential to a common chemistry and a common overall three-dimensional structure. The remaining amino acids are variable and adapt to different substrates to permit accurate substrate alignment. One class of enzymes also exists that has a common scaffold, but the enzymes catalyze different overall reactions, in which they utilize a common mechanistic strategy to facilitate the rate-limiting steps in the reactions they catalyze (Babbitt and Gerlt, 1997). These enzymes are believed to have evolved through the incorporation of new catalytic groups, in addition to the retaining of the necessary amino acid residues essential to all of their common chemistry. These features of enzyme evolution imply that it is possible to employ combinatorial saturation mutagenesis on the various amino acid residues close to active centers to introduce novel activities, and to explore substrate specificity of enzymes when the corresponding information is available.

Performing multiple-codon saturation mutagenesis, however, will generate libraries with huge numbers of variants. If saturation mutagenesis of interesting positions is achieved at the DNA level by an NNN randomization (N represents a mixture of A, C, T and G), single codon saturation mutagenesis will generate 64 variants, while double and triple codon saturation mutagenesis will produce 4096

and 262,144 variants, respectively. Numbers of variants increase rapidly, exceeding transformation efficiency (10^9 clones per μg of DNA) or screening performance (less than 10,000) when more codons are randomized. If some restrictions are applied to the codon randomization, the numbers of variants can be reduced, while the complexity of libraries can still be maintained. A common strategy is to utilize an NNG/C randomization rather than an NNN randomization. This will reduce the total number of variants, while all amino acids remain accessible. By using NTG/C, codon randomizations can be further restricted, leading to the generation of five hydrophobic amino acids: Phe, Leu, Ile, Met and Val. Alternatively, using NAG/C results in the production of seven hydrophilic residues: Tyr, His, Gln, Asn, Lys, Asp, and Glu. These methods might be useful for certain applications.

Table 1.1. Statistical analysis of libraries generated by NNG/C randomizations.

Number of codons to be randomized	Number of variants generated at DNA level	Number of variants generated at protein level	Number of variants required screening (at DNA level)*
1	32	20	95
2	1,024	400	3067
3	32,768	8,000	98,163
4	1,048,576	160,000	3,141,300
5	33,554,432	3,200,000	100,520,100
6	1,073,741,824	64,000,000	3,216,644,000

* Calculated using a Bernoulli distribution with 95% confidence level.

For most enzyme engineering efforts using combinatorial saturation mutagenesis, an NNG/C randomization strategy can be employed. The number of codon randomizations depends on the method used for isolating desired mutants. Table 1.1 lists the number of variants that must be screened for different numbers of codon randomizations. With an efficient genetic selection method, the maximal number of variants to be examined is determined by transformation efficiency. At most, six codon randomizations can be performed (Whittle and Shanklin, 2001). More than six codon randomizations will result in an incomplete examination of libraries. If a screening procedure is employed, three codon randomizations should be a realistic upper limit for combinatorial saturation mutagenesis.

3. Project Overview

This project focuses on the functional expression of GOase in *E. coli*, improvement of its total activity and thermostability in *E. coli*, and introduction of novel D-glucose 6-oxidase activities. Functional expression of mature GOase in *E. coli* is the first step to develop a high-through-put screening method for GOase activity, which is indispensable in the application of random mutagenesis or combinatorial saturation mutagenesis to engineer the properties of GOase. Functional expression of mature GOase in *E. coli* is also useful in understanding the biogenesis of the protein's thioether bond and the role of the precursor peptide in GOase folding and maturation. *E. coli*–expressed mature GOase is also a useful material to illustrate the biogenesis chemistry of thioether bond of

GOase, since it does not contain precursor peptide. Functional expression of mature GOase in *E. coli* is described and discussed in Chapter II.

GOase with increased activity and enhanced thermostability is expected to be advantageous for various applications. More active and more stable GOase can increase reaction efficiency in organic synthesis and decrease cost in the large-scale synthesis of carbohydrate compounds using GOase. More active and stable GOase can also improve the response and prolong the life of GOase-biosensors. Applications of more active and stable GOase will be effective approaches to enhancing the proficiency of GOase-containing reagent in medical research, food industry and many other applications. In Chapter II, the application of random mutagenesis, together with a high-through-put screening method to improve total activity and thermostability of GOase, is described and the resulting mutants are discussed. Both industrial and laboratorial applications will benefit from the increased activity, improved expression in *E. coli,* enhanced thermostability and decreased basicity of GOase.

GOase has excellent regioselectivity and stereoselectivity: it only oxidizes the 6-hydroxyl group of D-galactose and does not function on D-glucose, a 4-epimer of D-galactose. If we can expand the substrate range of GOase, while maintaining its regioselectivity for carbohydrate compounds, we may introduce novel activities desirable for various applications. A particular interesting novel activity is the selective oxidation of the 6-hydroxyl group of D-glucose, a functionality

never reported in nature or in the laboratory. D-Glucose is the most abundant of nature's products and its polymer forms are used widely in food, material and paper industries. Selective functionlization of the 6-hydroxyl group of D-glucose and its derivatives is expected to be valuable for modifying desirable properties, or introducing novel properties in D-glucose-containing materials. The engineering of GOase to a D-glucose 6-oxidase is also expected to be informative to the understanding of the physiological functions of carbohydrate oxidoreductases in fungi, commonly believed to generate hydrogen peroxide. In Chapter III, we introduce the novel glucose 6-oxidase activity using combinatorial saturation mutagenesis. The implications of the activities of carbohydrate oxidoreductases in fungi are discussed as well. In Chapter IV, biochemical characterizations and spectral properties of the mutant were described. The information revealed by the mutations on modeling and reaction mechanism of GOase are discussed.

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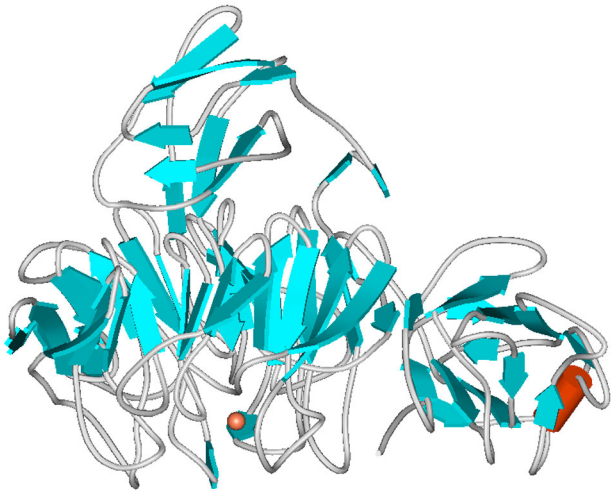

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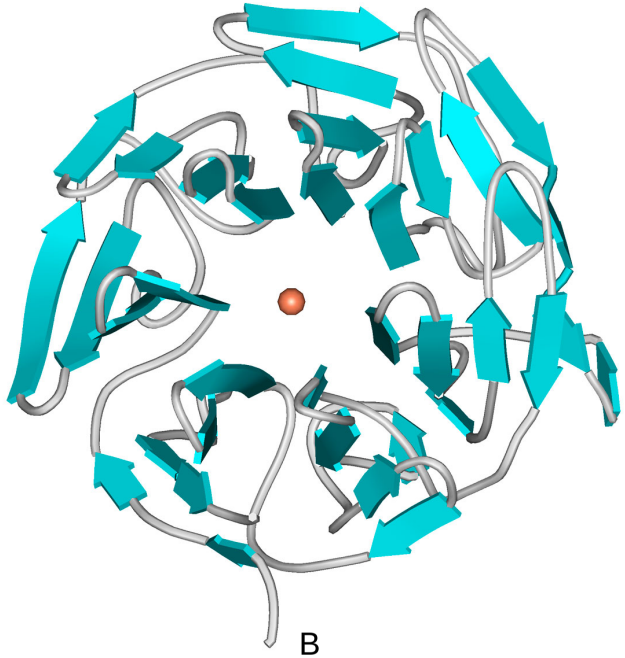

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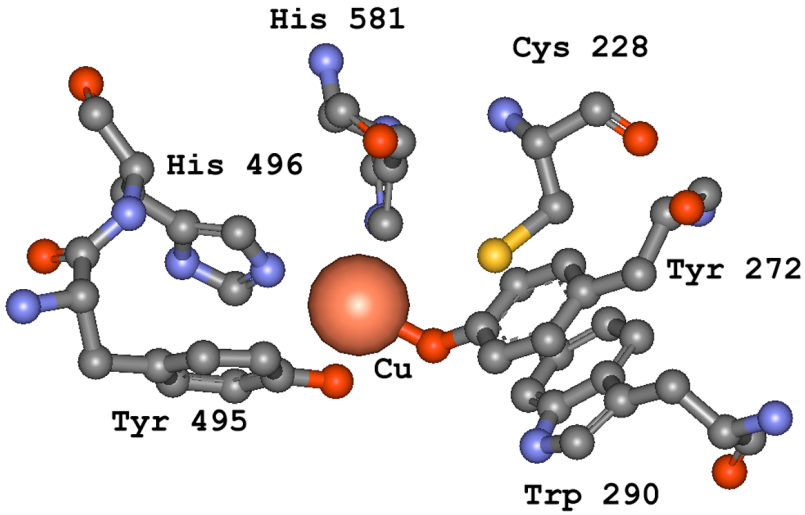

C

Figure 1.1. (A) Side view of the three–dimensional structure of GOase. (B) Top view of the second domain of GOase. (C) Active center residues and the radical position of GOase.

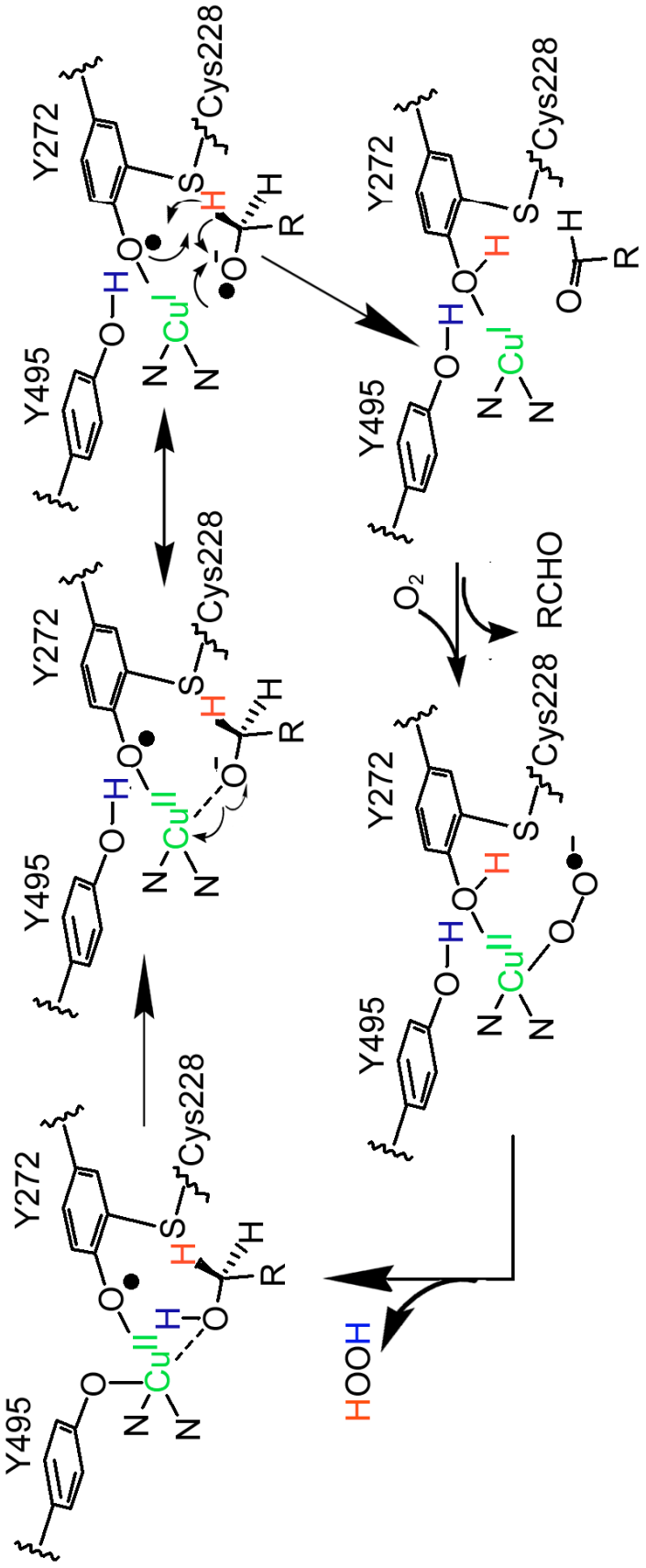

Figure 1.2. Proposed reaction mechanism.

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Chapter II

Directed Evolution of Galactose Oxidase Towards Increased Expression in *E. coli* and Enhanced Thermostability

(Portions of this chapter have been published: Sun, L., Petrounia, I. P., Yagasaki, M., Bandara, G. and Arnold, F. H. (2000). *Protein Engineering*, 14, 699-714.)

1. Introduction

During the last two decades, detailed biochemical, spectroscopic, and crystallographic characterization of GOase have generated abundant information regarding the enzyme's structure, kinetics, and reaction mechanism (Baron et al., 1994; Ito et al., 1994; McPherson et al., 1992; McPherson et al., 1993; Whittaker et al., 1989; Whittaker and Whittaker, 1988). All of these studies were performed with the GOase obtained from its native fungal species *Fusarium spp.* or an *Aspergillus nidulans* expression system. The GOase from these sources is fully functional; however, these fungal systems are inefficient at producing GOase. It usually takes several weeks to cultivate fungal cells to secrete sufficient amounts of functional GOase necessary for characterization. Due to the paucity of efficient genetic operation methods on fungi, it is difficult to apply various mutagenic techniques (Arnold, 1998) to engineer GOase to explore its biochemistry, reaction mechanism, and structure-function relationship. By expressing GOase in *E. coli*, all of these difficulties can be circumvented, allowing us to create mutant libraries and apply a high-throughput procedure to identify desired mutants in a reasonable amount of time. Functional expression of mature GOase in *E. coli* is also expected to be useful in illuminating the biogenesis of the thioether bond of GOase because of controllable bacterial cell cultivation conditions for generating copper-free and unprocessed GOase (Rogers et al., 2000). However, *E. coli* does not support functional expression of many eukaryotic enzymes, and functional expression of mature GOase in *E. coli* has never been achieved. A yeast *Pichia pastoris* expression system is capable

of generating a large amount of functional GOase (Whittaker and Whittaker, 2000), but it is not convenient for rapid and efficient genetic operations.

Functional expression of GOase in *E. coli* has been attempted (McPherson et al., 1993), but a functional enzyme was obtained only as a *lac*Z fusion (Lis and Kuramitsu, 1997) or with the precursor peptide (Delagrave et al., 2001). The fusion nature of the *E. coli*–expressed GOase is not suitable for biochemical characterization, nor further mechanistic research. In this chapter, and for the first time, the functional expression of mature GOase, without any precursor sequence, is reported and the resultant proteins are characterized.

We are also interested in improving the activities of GOase towards its natural or unnatural substrates and enhancing its thermostability. More active and stable GOase can increase reaction efficiency and decrease costs in organic synthesis, and improve the response and prolong the life of GOase-biosensors. Applying more active and stable GOase is an effective approach to enhancing the proficiency of GOase-containing reagents in medical research, food industry and other fields. In this chapter, we report the application of directed evolution to improve the total activity and thermostability of GOase. The final mutant, with an improved expression level in *E. coli*, enhanced thermostability, decreased basicity, and slightly increased specific activity, is advantageous for a variety of industrial and laboratorial applications.

2. Results and Discussion

2.1. Functional Expression of GOase in *E. coli*

The GOase gene from pR3 was amplified to introduce the ATG initiation codon, as well as the *Hind*III and *Xba*I restriction sites. The amplified fragment was subcloned into a pUC18 vector. Introduction of a second *lac* promoter increased expression of GOase and yielded plasmid pGAO-036. The cloned *gaoA* gene includes a precursor sequence encoding 41 amino acid residues. This sequence's N-terminal end is proposed to be involved with protein secretion and folding (McPherson et al., 1993) and is cleaved in a copper-mediated, self-processing reaction (Rogers et al., 2000). In this research, functional expression of mature GOase in *E. coli* was accomplished without the pro-sequence.

Expression of GOase with the precursor peptide in *E. coli* generated GOase of around 3% of the total protein (McPherson et al., 1993). Only a small fraction of GOase was active and could not be used for site-directed mutagenesis and crystallographic research. Since the expression of mature GOase in *E. coli* did not lead to a functional enzyme (McPherson et al., 1993), this suggests that the precursor peptide is necessary for correct folding of GOase in *E. coli*, though the sequence is not recognized for the secretion of GOase. The reducing nature of the cytoplasm of *E. coli* was also proposed to be responsible for inefficient formation of the protein's thioether bonds, resulting in the incorrect folding of GOase. Our expression results are in contrast with these observations. The active GOase expressed in *E. coli* was generated upon exposure to cupric ions.

Since it was not included in our expression system, the precursor peptide was demonstrated to be unnecessary for GOase folding. The identical migration rate of *E. coli*–expressed GOase and fungal GOase during SDS-PAGE indicates that the thioether bond is formed in *E. coli*–expressed GOase (Baron et al., 1994). This result shows that the formation of thioether bonds and cleavage of precursor peptides are independent processes, which were undistinguished in previous research (Rogers et al., 2000). It also verifies that the formation of the radical in GOase is an auto-catalytic, self-sufficient process (Rogers et al., 2000) independent of any other enzymes or fungal environments. The comparable kinetic parameters (Table 2.2) between *E. coli*–*expressed* GOase and native GOase indicate that the small contents of carbohydrates in native GOase (1%, 1-2 carbohydrate molecule) have no significant impact on the activity or folding of GOase.

However, the expression level of GOase in *E. coli* is low. Approximately 0.8 mg of purified protein were obtained from one litter of flask culture. Such a low level of protein is not convenient for crystallographic studies, since a large amount of purified protein is required. Meanwhile, GOase can be functionally expressed only when cells are grown at 30 ^{o}C or lower temperature. Low expression level and failure to generate functional GOase at high temperature might have discouraged researchers to further develop this expression system, but they allow us to develop a high-throughput screen to improve properties of GOase using directed evolution.

2.2. Development of a High-throughput Screening Method

A high-throughput screening method is indispensable when using directed evolution to engineer GOase. The reaction catalyzed by GOase can be monitored by either the consumption of oxygen or the generation of hydrogen peroxide. Since hydrogen peroxide can be detected conveniently by various colorimetric and fluorescent methods, we developed a high-throughput screen to detect the formation of hydrogen peroxide during the reaction. An ABTS-HRP (2,2' – Azino – bis (3 – ethylbenzothiazoline – 6 – sulfonic acid) and horseradish peroxidase) coupled assay, providing sufficient sensitivity to rapidly isolate superior mutants, was used to monitor the generation of hydrogen peroxide. Lysis of the *E. coli* cells to release GOase was achieved using lysozyme and SDS, taking advantage of the high stability of GOase against denaturation. The C.V. of the whole screening procedure (see material and experiment) was 15%, which was enough to identify more active mutants (usually 2-times improved activity) (Figure 2.1).

2.3. Directed Evolution of GOase

To increase the total activity of GOase in *E. coli*, random point mutagenesis was applied to the entire mature GOase gene (strategy A) and more locally to the region of the gene encoding domains II and III which are responsible for catalytic activity (strategy B) (McPherson et al., 1993). Adjusting the concentration of Mn^{2+} or Mg^{2+} during PCR led to an error rate of approximately 2-3 base substitutions per gene. Mutant libraries were screened for activity on D-galactose (in strategy

B), allyl alcohol, 3-pyridyl carbinol, and D-glucose (in strategy A), using horseradish peroxidase to detect the hydrogen peroxide produced during the reaction (Baron et al., 1994). Higher-activity mutants identified in each round were subjected to further mutagenesis or recombination.

Screening 1,600 mutants in the first round of mutagenesis of the complete GOase gene (library A1) generated two variants with 6- and 1.5-fold higher total activity toward D-galactose than the wild type (Table 2.1). The most active variant A1.D12 also showed enhanced thermostability and was used as a template for the second generation. Recombination of these two mutants did not lead to a more active or more stable mutant. Screening ~1,600 clones in the second generation identified four variants with improved activity and enhanced thermostability (A2.C3). Recombination of these four generated A3.E7 with approximately 60-fold higher total activity than wild-type GOase. No mutants with altered substrate specificity were identified.

Screening the first generation library created by random mutagenesis of domains II and III (library B1) produced variant B1.D4, which displayed approximately twice the total activity of wild type. Three more rounds of mutagenesis and screening yielded variant B4.F12 possessing approximately 15-fold higher total activity than wild type. Recombining the best mutants (A3.E7 and B4.F12) and screening ~3,200 clones yielded no further improvement in total activity.

2.4. Protein Purification, Kinetics, and Spectroscopy

A rapid two-step procedure consisting of fractionation by $(NH_4)_2SO_4$ and chromatography on Sepharose 6B was developed for the purification of GOase. Recombinant wild-type GOase and mutants B4.F12 and A3.E7 were purified and characterized. All three migrated on SDS-PAGE with an apparent molecular mass of 66 kDa instead of 68.5 kDa predicted by the DNA sequence. The faster migration rate indicates that the thioether bond between Cys228 and Tyr272 was formed in each enzyme sample (Baron et al., 1994; Rogers et al., 2000).

Kinetic parameters for fungal, *E. coli*–expressed wild type, and mutant GOases are reported in Table 2.2, as are the yields of purified enzymes. The fungal and wild-type recombinant GOases exhibit similar kinetic behaviors. Variant B4.F12 shows an 8-fold increase in the production of GOase at shake-flask level, while retaining the catalytic efficiency of the wild type. For variant A3.E7, the 30-fold increase in total activity, relative to wild type, reflects an 18-fold increase in GOase expression and a 1.7-fold increase in catalytic efficiency. This variant yields 10.8 mg/L of purified enzyme.

The broad substrate specificity of wild-type GOase (Avigad, 1985; Bretting and Jacobs, 1987) is retained in the evolved enzymes. Glycerol, xylitol, D-lactose and IPTG are oxidized by the wild type at 0.4%, 1.5%, 5% and 40% the rate of galactose oxidation, respectively. Similar relative activities were observed for the variant GOases. More substrates are examined in Chapter IV.

Purified GOase can exist in nature in three states: an oxidized state (active), a semireduced state (inactive) and a fully reduced state (inactive and unstable). Solutions of isolated pure GOase are a mixture of two oxidation states, oxidized (Cu^{2+}-$Tyr^{\bullet}$) and semireduced (Cu^{2+}-Tyr) (Whittaker and Whittaker, 1988). These states may be interconverted by treatment with oxidizing or reducing agents, and are distinguished by their absorption spectra: the semireduced form shows weak absorption over the visible region, whereas the oxidized form exhibits an intense absorption band at 445 nm and a broad band with a maximum near 810 nm (Baron et al., 1994; Whittaker and Whittaker, 1988). Treatment of wild type and evolved GOases with $K_3[Fe(CN)_6]$ at 4 ^{o}C generated the tyrosyl radical. The spectrum of the oxidized species persisted for several hours at ambient temperature (Figure 2.2), indicating that the oxidized form of the enzyme produced in *E. coli* has redox potential and stability comparable to fungal GOase (Baron et al., 1994; Reynolds et al., 1997). Oxidation and desalting of GOase at room temperature did not result in the oxidized state of GOase. This finding indicates that the radical quenchers in GOase solution can rapidly reduce the oxidized form of GOase to the semi-reduced state before or during desalting, at room temperature, but not at 4^{o}C. The failure to generate the oxidized state of GOase expressed in fungi *Fusarium venenatum* and *Aspergillus oryzae*, using the same oxidant, may occur for the same reason (Xu et al., 2000). This result supports the argument that the crystallization of GOase in the presence of oxidants does not always form an oxidized state crystal structure of GOase (Whittaker, 1994). The auto-decay process is 100% complete under oxygen-free

conditions, and involves a Cys515-Cys518 disulfide bond (Wright and Sykes, 2001).

2.5. Stability of GOase and Mutants

The thermostabilities of the purified fungal, *E. coli*–expressed wild-type, and mutant GOases are shown in Figure 2.3. The T_{50} (the temperature at which the enzyme loses 50% of its activity following incubation for 10 min) is increased to 67 ^{o}C for both B4.F12 and A3.E7 relative to a T_{50} of 63 ^{o}C for wild type. However, the *E. coli*–expressed wild type is less stable than the fungal enzyme, whose T_{50} is 67 ^{o}C. Similar results were obtained in the presence of 0.5 mM $CuSO_4$ (data not shown). A possible reason for the reduced thermostability of the recombinant wild-type enzyme may be due to its lack of glycosylation. It has been shown that the amount of carbohydrate compound in GOase is relevant to the thermostability of GOase. Extracellular GOase, with 7.7% of glycosylation, is more thermostable than intracellular GOase, with 1% of glycosylation. GOase with glycosylation between these two has a thermostability higher than extracellular GOase but less than intracellular GOase (Mendonca and Zancan, 1988). Combined with our kinetic results, we concluded that glycosylation in native GOase enables the enzyme to form a more rigid and stable structure, rather than enhance catalytic efficiency.

The stabilities of the enzymes have also been determined under conditions used in the synthetic applications of GOase – at room temperature in the presence of

$CuSO_4$ and catalase (Liu and Dordick, 1999; Mazur and Hiler, 1997; Root et al., 1985). As shown in Figure 2.3, variants B4.F12, A3.E7, and the fungal GOase are extremely stable, retaining ~80% activity following an 8-day incubation. By contrast, wild type has lost ~50% activity under these conditions. All these results indicate that the mutants have restored the compromised stability in *E. coli*–expressed GOase caused by the absence of glycosylation.

2.6. Sequences of Evolved GOases

The mutations identified in the most highly expressed GOase variants are listed in Table 2.1. The amino acid substitutions S10P, M70V, and N413D, as well as the synonymous mutations in codons S550 and S610 contribute to enhanced enzyme expression. The V494A substitution increased both expression (variant B3.H7) and thermostability (variant A1.D12). G195E is a thermostabilizing mutation that appeared in clone A2.C3. When recombined with other mutations in generation A3, the advantageous effect of N535D on expression became evident.

Identifying the mechanisms responsible for the increased expression is difficult. However, we can provide some speculation. The mutation leading to amino acid substitution S10P is located at the N-terminal of GOase gene; the nucleotide sequence in this region strongly influences gene transcription (Boer and Hui, 1990). Mutations could also contribute to changes in the secondary structure of GOase mRNA, which can affect protein expression (Cheong and Oriel, 2000).

Synonymous mutations S550 and S610 generate codons that are much less frequently used (TCT, 17.4 per thousand to TCA, 1.0 per thousand). Using rare codons in specific regions of the gene can be advantageous for protein expression (Komar et al., 1999), probably by inducing pauses in translation which result in a slower rate of protein synthesis and decreased levels of protein misfolding.

Screening the library of recombining mutants A3.E7 and B4.F12 did not identify a more active variant than A3.E7. Nor did the introduction of the N413D substitution into mutant A3.E7 by site-directed mutagenesis foster a more active clone, demonstrating that the mutation effects are not cumulative.

Figure 2.5 shows the positions of the amino acid substitutions in variants B4.F12 and A3.E7. The V494A substitution is adjacent to the Cu(II) ligands Tyr495 and His496. The thermostabilizing mutation G195E occurs in a loop at the active site entrance, 10 Å away from copper. In wild type, Gly195 forms a hydrogen bond (2.90 Å) with Tyr189. A second, charged hydrogen bond to Gly196 (2.40 Å) is introduced upon replacement of Gly with Glu, which may explain the beneficial influence of this mutation on GOase stability.

Three of the mutations identified in the evolved GOases (Figure 2.6) result in the replacement of neutral residues with negatively charged residues at the surface of the enzyme. Introduction of the negative charges reduces the unusually high

basicity of the enzyme (pI=12) (Mazur, 1991) and is speculated to weaken interactions with other macromolecules. This will attenuate the sensitivity of GOase to inhibition and inactivation by macromolecular contaminants as well as promote reactivity with glycoconjugates attached on the cell membranes, (Mazur, 1991). This effect, along with the thermostabilizing influence of V494A, need not be confined to the *E*. *coli* host system. Since these mutations are not detrimental to catalytic efficiency (Table 2.1), introduction of these amino acid substitutions in GOase and expression of the enzyme in high-level expression hosts, such as *Pichia pastoris* (Whittaker and Whittaker, 2000) and *Aspergillus oryzae* (Xu et al., 2000), should generate active GOase of reduced basicity and increased stability. Enhanced thermostability and improved expression in *E. coli* facilitate protein purification and characterization. Enhanced expression also improves the reproducibility and sensitivity of screening. Thus the evolved GOase will be an advantageous template for further evolution. Functional expression of mature GOase in *E. coli* will facilitate and simplify genetic operations on the GOase gene, providing a reliable and easily accessible source of GOase for reaction mechanism research and thioether biogenesis studies.

3. Materials and Methods

3.1. Materials

All chemicals were reagent grade or better. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), D-galactose and horseradish peroxidase (HRP) were from Sigma (St. Louis, MO). Native *Fusarium* galactose oxidase was obtained

from Worthington Biochemical Corporation (Lakewood, NJ). *E. coli* strain BL21(DE3) and vector plasmid pUC18 were purchased from Novagen (Madison, WI). Restriction enzymes and ligase were obtained from Boehringer Mannheim (Indianapolis, IN), Life Technologies (Grand Island, NY) or New England Biolabs (Beverly, MA).

3.2. Bacterial Strain and Plasmids

Bacterial strain BL21(DE3) was used for cloning and library construction. Plasmid pR3 containing the gene for mature GOase fused to the 5′ end of the *lac*Z fragment was kindly provided by Dr. Howard K. Kuramitsu (Department of Oral Biology, State University of New York at Buffalo). The GOase gene was amplified from pR3 by PCR to introduce a *Hin*dIII restriction site followed by an ATG initiation codon immediately upstream from the mature GOase sequence and *Xba*I site immediately downstream from the stop codon. The PCR product was subcloned into a modified vector pUC18 (containing a double *lac* promoter and lacking the *Pst*I site) to yield pGAO-036.

3.3. Construction of GOase Mutant Libraries

Galactose oxidase was expressed in *E. coli* using plasmid pGAO-036. Two approaches were followed for directed evolution: (A) random mutagenesis of the complete GOase gene (bases 1-1917) by error-prone PCR (generations A1 and A2) and StEP recombination of improved variants from library A2 (generation A3)

and (B) sequential random mutagenesis of a region of the GOase gene (bases 518-1917) by error-prone PCR (generations B1 through B4).

3.3.1. Random Mutagenesis and StEP Recombination of the Complete GOase Gene

Error-prone PCR and StEP recombination (Zhao *et al.*, 1998) were carried out using primers 5′-AATTCGAAGCTTATGGCCTCAGCACCTATCGGAAGC-3′ (*Hin*dIII site underlined) and 5′- CCTCCTTCTAGATTACTGAGTAACGCGAAT CGT-3′ (*Xba*I site underlined). The mutagenic PCR reaction contained 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.5 at 25 ^{o}C), approximately 0.3 μg plasmid DNA as template, 30 pmol of each primer, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 7 mM $MgCl_2$, 0.1 mM $MnCl_2$ and 1.5 U *Taq* polymerase (Perkin-Elmer, Gaithersburg, MD or Qiagen, Valencia, CA) in a total volume of 100 μl. PCR reactions were carried out on a MJ Research (Watertown, MA) thermal cycler (PTC-200) for 30 cycles with the following parameters: 94 ^{o}C for 30 seconds, 50 ^{o}C for 30 seconds and 72 ^{o}C for 60 seconds. StEP recombination of four improved variants identified in generation A2 was performed in a 100 μl reaction containing 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.5), approximately 0.3 μg (total) plasmid DNA as template (prepared by mixing equal amounts of the four plasmids), 10 pmol of each primer, 0.5 mM of each dNTP, 2.5 mM $MgCl_2$ and 5 U *Taq* polymerase. PCR conditions were as follows: 95 ^{o}C for 3 minutes and 100 cycles of 94 ^{o}C for 30 seconds and 58 ^{o}C for 10 seconds. Mutagenic PCR or recombination products were purified using a DNA purification kit

(Qiagen or Zymo Research, Orange, CA) and cloned (using the *Hin*dIII and *Xba*I restriction sites) back into the expression vector. Ligation mixtures were transformed into BL21(DE3) cells by electroporation.

3.3.2. Random Mutagenesis of GOase Gene Region 518-1917

Error-prone PCR was carried out using primers 5′- TTGTTCCTGCGGCTGCAG CAATTGAACCG-3′ (*Pst*I site underlined) and 5′- TGCCGGTCGACTCTCTAGA TTACTGAGTAACG-3′ (*Xba*I site underlined). Mutagenic PCR was performed in a 100 μl reaction mixture containing 10 mM Tris-HCl, 50 mM KCl buffer (pH 8.3 at 25 ^{o}C), 10 ng plasmid DNA as template, 50 pmol of each primer, 0.2 mM of each dNTP, 7 mM (generation B1 and B2) or 4 mM $MgCl_2$ (generation B3 and B4) and 5 U *Taq* polymerase (Boehringer Mannheim). PCR conditions were as follows: 94 ^{o}C for 2 minutes and 25 cycles of 94 ^{o}C for 30 seconds, 58 ^{o}C for 30 seconds and 72 ^{o}C for 60 seconds. Purified restricted inserts from PCR reactions were ligated with an expression vector generated by *Pst*I-*Xba*I digestion of pGAO-036. Ligation products were transformed into BL21(DE3) cells by a modified chemical transformation method (SuperComp protocol, Bio 101, Inc., Carlsbad, CA)

3.4. Screening GOase Libraries

Transformed cells were plated on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin and grown overnight at 30 ^{o}C.

3.4.1. Screening of Libraries A1 - A3

Single colonies were picked into deep-well plates (well depth 2.4 cm; volume 1ml; Beckton Dickinson Labware, Lincoln Park, NJ) and cells were grown for 10 h at 30 ^{o}C and 270 rpm in 200 µl LB containing 100 µg/ml ampicillin (LB-Amp). The master plates were duplicated by transferring a 10 µl aliquot to a new deep-well plate containing 300 µl LB-Amp and 1 mM IPTG and grown for 12 h at 30 ^{o}C and 250 rpm. The cultures were then centrifuged for 10 minutes at 5000 rpm and the cell pellet was resuspended in 300 µl 100 mM sodium phosphate (NaPi) buffer, pH 7.0, containing 0.4 mM $CuSO_4$. Following addition of 0.5 mg/ml lysozyme (35 min at 37 ^{o}C) and 2.5% (w/v) SDS (overnight at 4 ^{o}C), the GOase activity was assayed using a GOase-HRP coupled assay (Baron *et al.*, 1994). Aliquots of the cell extracts were reacted with D-galactose, allyl alcohol, 3-pyridyl carbinol and D-glucose at pH 7.0. The initial rate of H_2O_2 formation was followed by monitoring the HRP-catalyzed oxidation of ABTS at 405 nm on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). Thermostability was assayed as follows: aliquots of the cell extracts were heated at 55–70 ^{o}C for 10 minutes and then chilled on ice for 10 minutes. The samples were equilibrated to room temperature, at which point they were assayed for activity. The ratio of residual to initial activity was used to characterize thermostability. 1500-2000 clones from each library were screened and the clones with improved activity or enhanced thermostability accompanied with activity comparable to the parents were picked for further verification.

3.4.2. Screening of Libraries B1 - B4

Single colonies were picked into deep-well plates (well depth 4.4 cm; volume 2.2 ml; Qiagen) and cells were grown for 8 h at 30 ^{o}C and 270 rpm in 500 µl LB-Amp. The master plates were duplicated by transferring a 10 µl aliquot to a new deep-well plate containing 500 µl LB-Amp-1mM IPTG and grown overnight at 30 ^{o}C and 270 rpm. An aliquot of the culture was transferred to a microtiter plate. Following addition of 0.5 mg/ml lysozyme (30 min at 37 ^{o}C) and 0.4% (w/v) SDS-0.4 mM $CuSO_4$ in 100 mM NaPi buffer, pH 7.0 (4 h at 4 ^{o}C), the GOase activity was assayed using the GOase-HRP coupled assay as described above. The galactose concentration was 25 mM (generations B1 and B2) or 10 mM (generations B3 and B4). Approximately 1000 clones from each library were screened.

3.5. Protein Purification and Characterization

E. coli cultures were grown for 16 hours at 30 ^{o}C in LB medium with 100 µg/ml ampicillin. Cells were harvested by centrifugation, resuspended in 100 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and disrupted by sonication. Cell debris was removed by centrifugation and the resulting supernatant was made 0.4 mM in $CuSO_4$ and stirred for approximately 2 hours at 4 ^{o}C. $(NH_4)_2SO_4$ was added to 25% saturation (w/v) and after centrifugation the supernatant was further saturated to 65% of $(NH_4)_2SO_4$. The pellet was dissolved in 100 mM CH_3COONH_4 buffer, pH 7.2, and chromatographed on Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) according to published

procedures (Hatton and Regoeczi, 1982). Fractions with the highest GOase activity were collected and precipitated by addition of $(NH_4)_2SO_4$ to 95% saturation. The pellet was dissolved in 100 mM NaPi, pH 7.0, and dialyzed extensively against the same buffer (the first dialysis buffer contained 0.2 mM $CuSO_4$). The dialyzed protein was filtered through a 0.2 μm filter and frozen immediately at –80 ^oC. Fungal GOase was purified by passage through a Sepharose 6B column. The eluted protein was incubated with 0.1 mM $CuSO_4$ for 4 h at 4 ^oC and desalted using a Biogel column (Bio-Rad, Hercules, CA).

The purified protein ran as a single band during SDS-PAGE (Novex, San Diego, CA). Protein concentrations were determined from the absorbance at 280 nm ($\in = 1.05 \times 10^5\ M^{-1}cm^{-1}$) (Ettinger, 1974). Kinetic measurements were performed in 100 mM NaPi buffer, pH 7.0, over a range of D-galactose concentrations from 15 to 250 mM using the HRP-ABTS coupled assay (Baron et al., 1994). The rate of absorbance change was monitored by a Shimadzu (Columbia, MD) UV-Vis spectrophotometer at 420 nm ($\in = 42.3 \times 10^3\ M^{-1}cm^{-1}$) (Yamazaki et al., 1997).

UV-Vis spectra of wild-type and mutant GOases were recorded from 320 to 900 nm. Oxidation of GOase was performed in 100 mM NaPi, pH 7.0, by incubation with 100 mM $K_3[Fe(CN)_6]$ for 10 min followed by removal of the oxidant on a Biogel column at 4 ^oC (Whittaker and Whittaker, 1988).

Thermostability of the purified native, wild-type and mutant GOases was assessed by measuring residual activity/initial activity over the temperature range 24-75 ^{o}C. Enzymes (30 µl each, 0.054 mg/ml) were incubated at each temperature for 10 minutes in a MJ Research thermal cycler and chilled on ice before they were assayed in a microtiter plate. Stability at ambient temperature was measured by incubating 0.04 mg/ml GOase in the presence of 0.5 mM $CuSO_4$ and 3 units of catalase in a total volume of 100 µl and measuring activity as a function of time.

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Table 2.1. Mutants identified during directed evolution of GOase.

Generation	GOase Name	Base Substitutions	Mutations Identified[(a)]	Relative Total Activity for D-Galactose
0	Wild type			1.0
A1	A1.D2	A1609G	N537D	1.6
A1	A1.D12	T408C/T1481C	*P136*/V494A	5.9
A2	A2.D3	T408C/T1481C/T28C	*P136*/V494A/ S10P	11.1
A2	A2.C3	T408C/T1481C/A9C/ G584C	*P136*/V494A / *A3*/G195E	9.1
A2	A2.D6	T408C/T1481C/T654C/ A936G/A1603G	*P136*/V494A/*T218*/ *L312*/N535D	11.7
A2	A2.E12	T408C/T1481C/A208G	*P136*/V494A/M70V	16.4
A3	A3.E7	T408C/T1481C/T28C/ A208G/G584A/A1603G	*P136*/V494A / S10P/M70V / G195E/N535D	63.4
B1	B1.D4	A1237G	N413D	2.4
B2	B2.G4	A1237G/T1650A	N413D/*S550*	3.8
B3	B3.H7	A1237G/T1650A/ T1481C	N413D/*S550*/ V494A	10.5
B4	B4.F12	A1237G/T1650A/ T1481C/T1830A	N413D/*S550* / V494A/S610	15.2

(a) Synonymous mutations are indicated by italics.

Table 2.2. Yields and kinetic parameters of purified native fungal, wild-type and mutant GOases.

GOase	Yield (mg/L)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/ K_m ($M^{-1}s^{-1}$)
Native GOase		95 ± 8	1400 ± 120	1500 ± 1700
Wild type	0.6	57 ± 3	1100 ± 40	1900 ± 1200
B4.F12	4.7	57 ± 3	1200 ± 80	21000 ± 1700
A3.E7	10.8	43 ± 2	1400 ± 40	32000 ± 1700

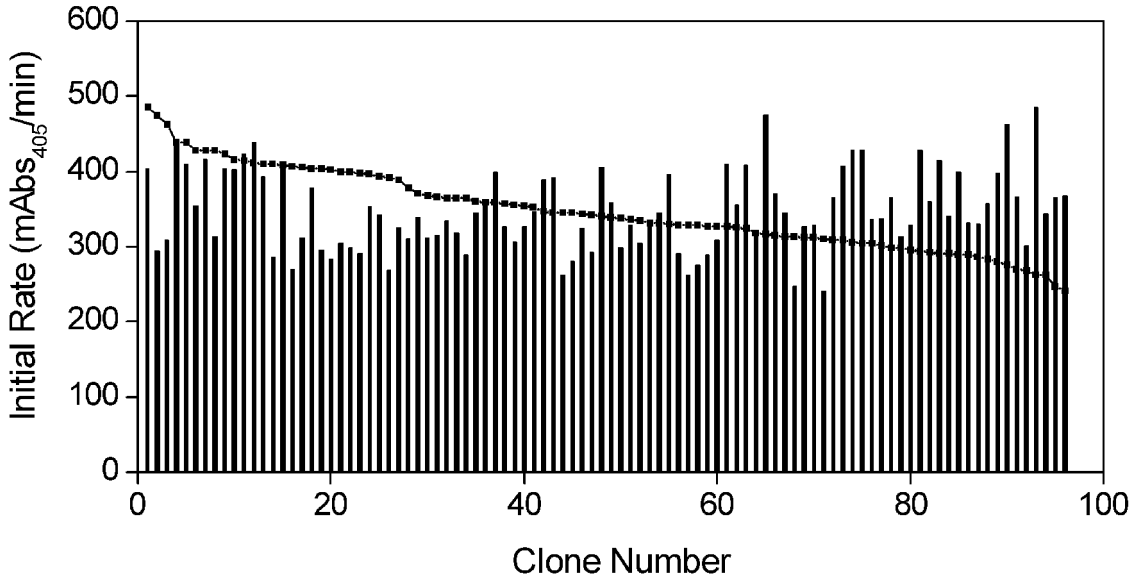

Figure 2.1. Validation of the screening method used in identifying superior mutants. Wild-type GOase was used to determine the reproducibility of the screening method. The column graph shows that the initial rate changes with the position of clone in a 96-well plate. The initial rate is also plotted in a decending order.

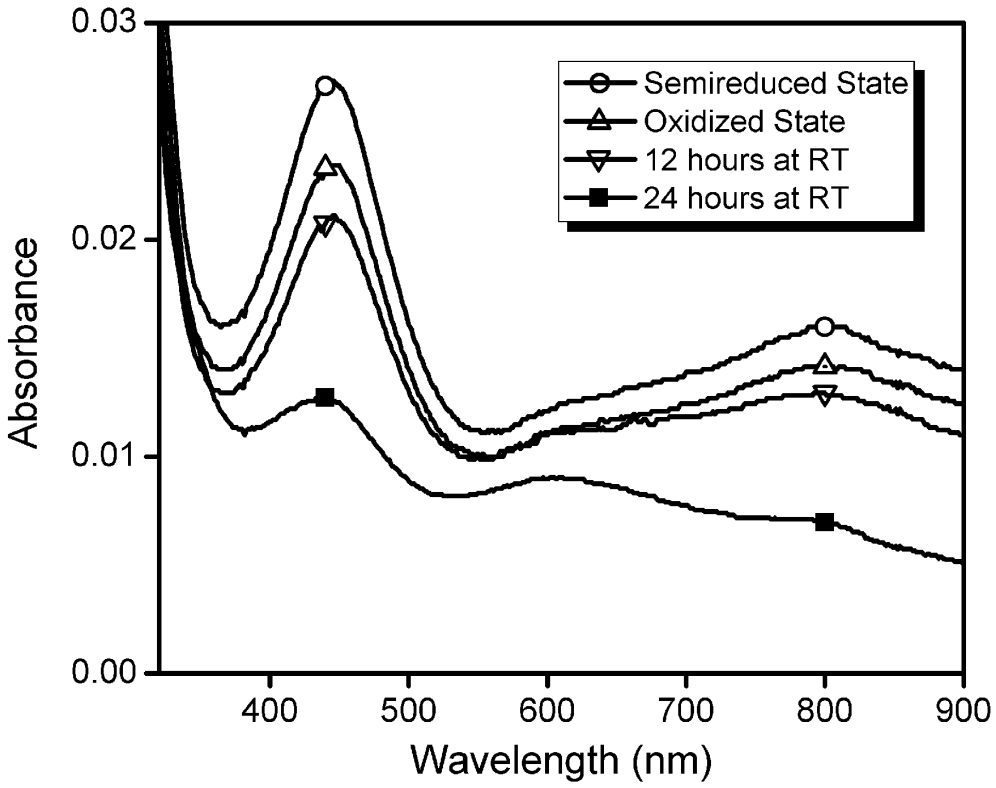

Figure 2.2. Optical absorption spectra of 16 μM A3.E7 GOase in the semireduced and oxidized states. Spectra of oxidized GOase were recorded immediately and at 12 h and 24 h following redox-activation by $K_3[Fe(CN)_6]$.

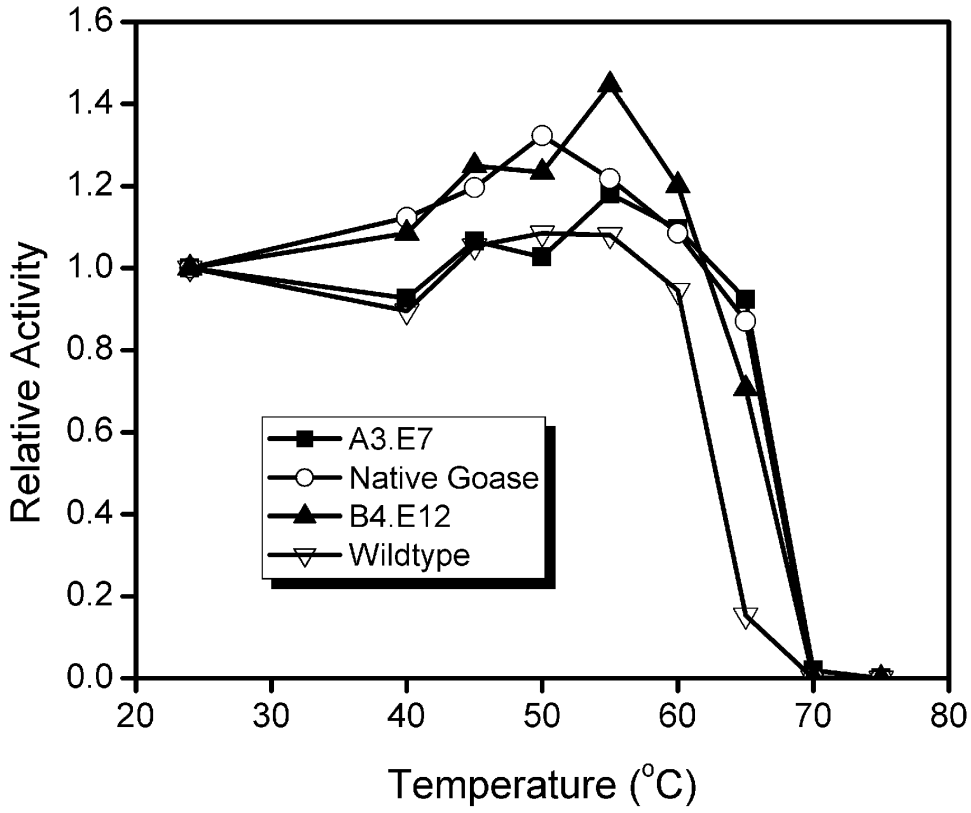

Figure 2.3. Thermostability of wild-type, mutant and native GOases. Enzyme samples (1.6 μg) were assayed for thermal stability (residual activity/initial activity) after incubation for 10 min in 100 mM NaPi, pH 7.0, at a temperature range between 24 and 75 ^{o}C.

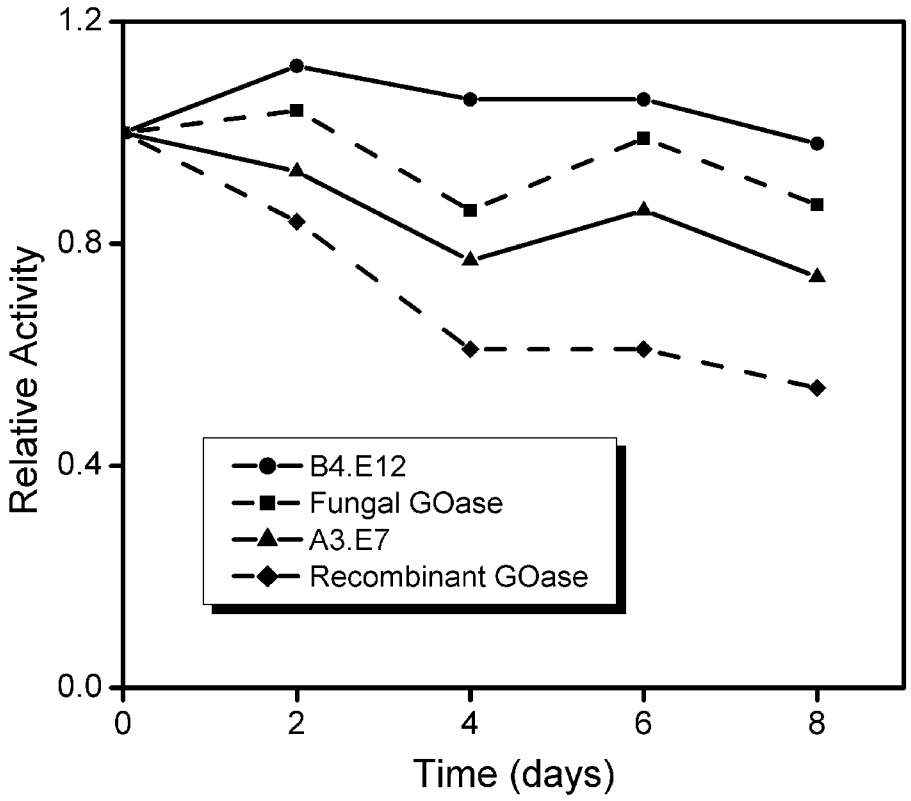

Figure 2.4. Long-term stability of the native and *E. coli*–expressed GOases. 4 μg of each enzyme were incubated at room temperature in the presence of 0.5 mM $CuSO_4$ and 3 units of catalase in 100 μl of NaPi buffer (0.1 M, pH 7.0).

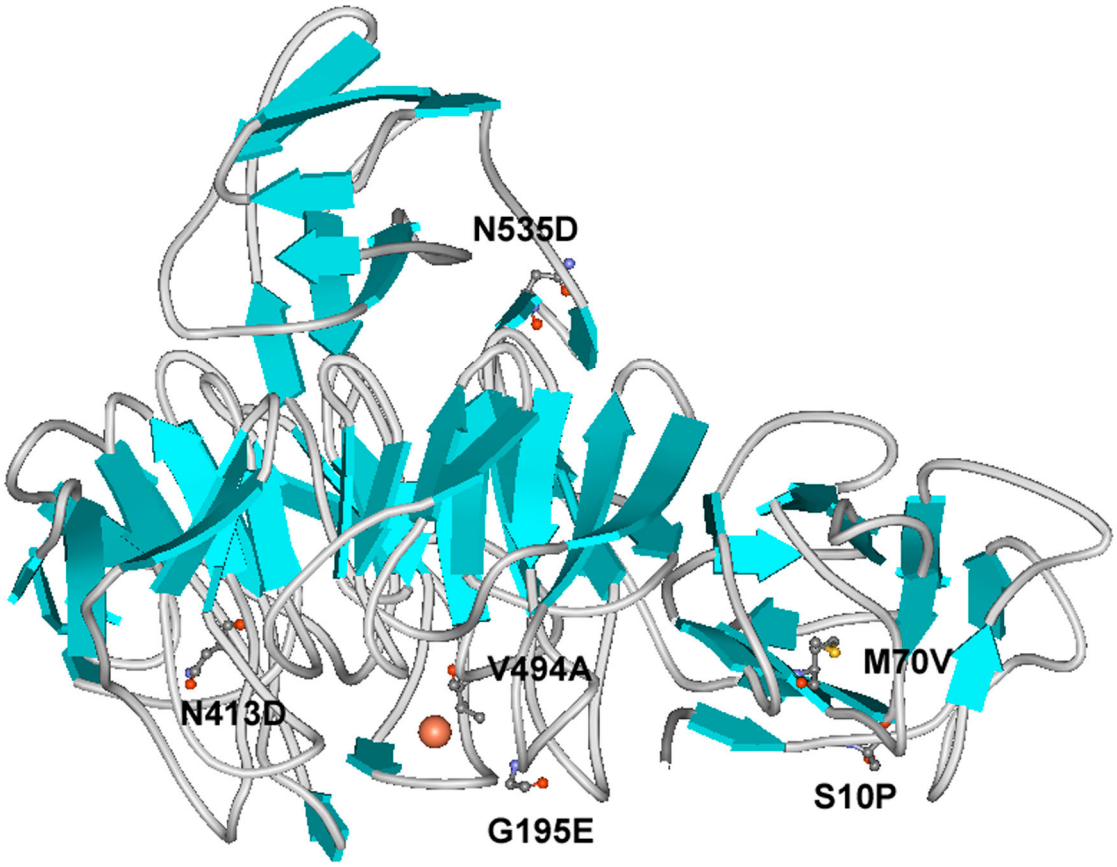

Figure 2.5. Amino acid substitutions identified in GOase variants A3.E7 and B4.F12. Mutations are illustrated in ball-and-stick form. Inset shows the copper ion and residues comprising the GOase active site.

Chapter III

Introduction of D-glucose 6-Oxidase Activity into Galactose Oxidase

(Portions of this chapter have been published: Sun, L., Bulter, T., Alcalde, M., Petrounia I. P., and Arnold, F. H. (2002). *ChemBioChem*, 3, 781-783)

1. Introduction

Oxidoreductases of free carbohydrates (nonphosphorylated) are present in a wide range of fungal species. The occurrence of galactose oxidase and glucose oxidase is suggested to be a widespread property in fungi (Gancedo et al., 1966). The characteristic feature of these fungal systems is the enzymatic production of dicarbonyl carbohydrate derivatives, whose physiological roles and metabolic fates remain unknown. The byproduct hydrogen peroxide has been proposed to serve as the cosubstrate of intracellular peroxidases to facilitate the lignin degradation, based on the discovery that sugar oxidoreductases are the main sources of hydrogen peroxide production in some species of white rot fungi (Ander and Marzullo, 1997). It was suggested that lignin degrading white-rot fungi may use different carbohydrate oxidoreductases to regulate the lignin degradation, depending on the cultivation conditions (Ander and Marzullo, 1997). Carbohydrate oxidoreductases are also generated and inhibited at the different growing stages of fungi (Gancedo et al., 1966). The regulation of carbohydrate oxidoreductase production suggests that the toxicity of carbohydrate oxidoreductases is well controlled in fungi.

Various enzymes involved in the oxidation of D-glucose, glucose 1-oxidase, glucose 2-oxidase (pyranose oxidase) and glucose dehydrogenase (pyranose 2, 3-dehydrogenase), have been isolated and characterized (Figure 3.1) (Giffhorn, 2000; Volc et al., 2001; Volc et al., 1998). Moreover, enzymes oxidizing oligoglucose have been identified (Cameron and Aust, 2001; Xu et al., 2001).

However, the enzymatic oxidation of the 6-hydroxyl group of D-glucose, generating the corresponding dicarbonyl derivative, is unknown. There is evidence that *Pseudogluconobacter saccharoketogenes* can produce an enzyme to oxidize the hydroxymethyl group of the terminal glucose residue of a cyclomaltooligosacchride, but the oxidation product is a carboxylic acid (Ishiguro et al., 2001). It is interesting that fungi do not utilize glucose 6-oxidoreductase to achieve the functionalities of oxidoreductases to generate hydrogen peroxide with the widely spread substance D-glucose and its derivatives. However, galactose oxidase (galactose 6-oxidase) (Amaral et al., 1963) can be found widely in fungal species, indicating that the C6-oxidation activity of carbohydrates is not prohibited in nature. Despite the relatively relaxed substrate specificity of carbohydrate oxidoreductases, the absence of glucose 6-oxidase activity suggests that glucose 6-oxidoreductase might not confer adaptive fitness to fungi.

We are interested in creating this rarely occurring glucose 6-oxidase activity with enzyme engineering. Galactose oxidase selectively oxidizes the 6-hydroxyl group of D-galactose, but its activity towards D-glucose, a 4-epimer of D-galactose, has never been reported. By changing its stereoselectivity, GOase might be an interesting starting material for the creation of glucose 6-oxidase activity.

It is also of interest to selectively oxidize the 6-hydroxyl group of D-glucose and its derivatives to introduce a carbonyl group in synthetic applications. D-Glucose

containing polysaccharides are ubiquitous in nature and are important materials in the food and paper industries. Introducing a carbonyl group, at C-6 of a D-glucose residue, will facilitate the functional diversification of polysaccharides, therefore enhancing the functions and generating new properties. Because of the numerous functional groups in a polysaccharide, it is expected to be advantageous to regioselectively and stereoselectively oxidize a desired hydroxyl group with an enzyme. Therefore, an enzyme selectively oxidizing the 6-hydroxyl of D-glucose will be useful and valuable in modifications of polysaccharides.

We have applied directed evolution to improve the expression level and thermostability of GOase in *E. coli* (Chapter II), meanwhile, we also screened activity of the libraries for D-glucose. However, no mutants with preferred activity towards D-glucose were identified. Here, we report the introduction of the glucose 6-oxidase activity into GOase through saturation mutagenesis.

2. Results

2.1. Library Construction and Screening

A slightly modified screening procedure based on the previous method (Chapter II) was utilized. More cell extracts were used to screen activities towards D-glucose. The CVs of the screening procedures for D-galactose activity and D-glucose activity are 14% (kinetic assay) and 21% (endpoint assay) (Figure 3.2).

The three-dimensional structure of GOase has been solved and a substrate binding model has been proposed, which is based on the findings of a molecular docking experiment (Baron et al., 1994; Ito et al., 1991). In the substrate binding model, the active center's water-accessible surface is consistent with the chair-form of D-galactose. It suggests that residue Arg-330 (Figure 3.3) forms hydrogen bonds with the C(4) and C(3) hydroxyl groups of D-galactose, while residue Gln-406 forms an additional hydrogen bond with the hydroxyl group of C(2). On the other hand, a hydrophobic wall along the pocket containing Phe-194 and Phe-464 residues interacts with the backbone (C(6), C(5) and C(4)) of D-galactose. Residue Trp-290, which has been proposed to stabilize the radical of GOase (Baron et al., 1994), is believed to have a key role in restricting entry to the active center. The substitution of Trp-290 with a histidine increased the active center's accessibility to buffers (Saysell et al., 1997). We expected the replacement of these residues to alter the substrate binding preference of GOase.

A saturation mutagenesis library (Lib-RFQ) was constructed by repeating codon randomizations on residues Arg-330, Phe-464 and Gln-406 using A3.E7 (see Chapter II) as the template. The library can produce approximately 8,000 variants at the amino acid level or over 30,000 variants at the nucleotide level. More than 10,000 clones were screened for their activities towards D-galactose and D-glucose, and the two best mutants (M-RQ: R330K, Q406T and M-KS: R330K, Q406S), with 20 times increased activity towards D-glucose and 100 times decreased activity towards D-galactose were identified. Mutants containing the mutation R330K were also isolated and showed 10 times increased activity with D-glucose. More than 95% of the screened clones were inactive. No clones exhibited higher D-galactose activity than the parent. On the other hand, only a few clones showed D-galactose activity comparable to the parent.

One more round of saturation mutagenesis on the residue F195, using the mutant M-RQ and M-KS as the templates, was performed. However, no mutants with improved activity towards D-glucose were identified. More than 50% of the clones were active, although none of them were more active than the parent for both D-galactose and D-glucose. This suggests that the hydrophobic interactions between F195, and probably F464, with substrates are important for correct substrate alignment at the active center.

The saturation mutagenesis of the residue W290 was performed, and one mutant (M-W, W290F) with 10 times improved D-glucose activity and 30 times decreased

D-galactose activity was identified. No mutants with enhanced D-galactose activity were isolated. The introduction of the mutation W290F into the mutant M-RQ resulted in a mutant (M-RQW) with 100 times greater D-glucose activity and 1000-fold diminished D-galactose activity.

2.2. Identification of Oxidation Product Generated by Mutant M-RQW

We developed an ion-exchange column-based protein purification procedure according to the published method to purify mutant M-RQW (see Chapter IV). The purified enzymes were used to catalyze the oxidation of methyl-β-D-gluco-pyranoside to identify the reaction product. Another well-established reaction, the oxidation of methyl-β-D-galacto-pyranoside by native GOase, was performed to examine product isolation methods and facilitate the assignment of the reaction product NMR spectra.

TLC was used to monitor and isolate the reaction product using chloroform-methanol (4:1), as the mobile phase, on a silica gel plate. Both reaction products can be isolated, with good resolution (Figure 3.4). Using this method, several by-products (less than 10%) were observed in both reactions which were proposed to be the results of the dimerization and α-β elimination of the reaction product (Maradufu and Perlin, 1974; Singh et al., 1989) (Figure 3.5). The TLC plates were also subjected to an aldehyde assay using Purpald reagent. Excluding the substrates, all components were stained. The reaction mixture was loaded into a silica gel column equilibrated with the mobile phase (chloroform-methanol) and

eluted with the same solvent at room temperature. The isolated reaction product was checked on a TLC plate. All of the components in the reaction mixture, except the substrates, were detected and stained by Purpald reagent. These results indicate that the by-products are generated from the reaction products nonenzymatically. More than 90% of the isolated products are the desired aldehydes.

^{13}C NMR spectra of two reaction products showed very similar chemical shifts for all the carbon atoms. Compared to the substrate spectra, the chemical shift of C6, in both products, changed from 61 ppm to 89 ppm (Figure 3.6 and 3.7), confirming the formation of an aldehyde, which is generated by the oxidation of the 6-hydroxyl of the substrate. ^{1}H NMR spectra also revealed the formation of an aldehyde (hydrated) through a signal with a chemical shift of 5.25 ppm (Figure 3.8 and 3.9).

There are no detectable signals in the NMR spectra for ketones, nor measurable amounts of ketone products on TLC plates. In addition, the oxidation product of D-glucose with mutant M-RQW showed that there was one main product, checked by TLC. The oxidation of the 1-hydroxyl group was, therefore, not detectable. These results indicate that the oxidation of the 6-hydroxyl of D-glucose by the mutant M-RQW is specific, and no other hydroxyl groups can be oxidized to generate measurable amounts of products.

3. Discussion

The substrate specificity of an enzyme can be altered by rational design (Broun et al., 1998; Xiang et al., 1999) or directed evolution (Matsumura and Ellington, 2001; Yano et al., 1998). The use of random mutagenesis, in concert with high-throughput screening methods in directed evolution, enables us to change the substrate preference of enzymes without any knowledge of structural information. More importantly, the beneficial mutations, far from the enzyme's active center, can be explored and accessed with directed evolution (Que et al., 1999).

We applied random point mutagenesis to improve the activity of GOase towards D-glucose. However, no mutants with preferred activity towards D-glucose were identified in the screening of more than 30,000 clones. Although, increased expression level and enhanced thermostability were achieved (see Chapter II). The failure to alter the substrate specificity of GOase with random point mutagenesis may indicate that GOase's substrate binding configuration is difficult to be changed by single mutations. The detailed kinetic research revealed that wild-type GOase is one million times less active on D-glucose than on D-galactose. Such low activity requires a very sensitive and reproducible screening method for further improvement with random point mutagenesis.

Combinatorial saturation mutagenesis was utilized in this research to introduce the D-glucose activity into GOase. None of the mutants are more active on D-galactose, indicating that the three residues (Arg-330, Phe-464 and Gln-406) are

crucial and optimal for D-galactose binding. Since the mutant with the highest activity on glucose contains two mutations (R330K and Q406T/S), these two positions were revealed to be important in maintaining the binding of D-galactose and D-glucose, as proposed by the binding model. The identification of mutations Q406T/S implies that the –OH group might be necessary in this amino acid residue, to form a hydrogen bond with D-glucose and facilitate its entry into the active center. The mutant with one mutation R330K, with 10 times increased activity on D-glucose, was isolated from the library. However, the mutant with single mutation Q406T/S was not found. This indicates that the mutation Q406T/S alone does not have sufficient beneficial effects on D-glucose activity to be isolated. The increased D-glucose activity of the mutant M-RQ and M-KS, 100-fold more active than the parent, therefore, results from the synergistic effects of the two mutations.

The important role of residue W290 in GOase catalysis has been illustrated by site-directed mutagenesis (Saysell et al., 1997). The replacement of the tryptophan for a histidine (W290H) increased the accessibility of the active center and also increased the redox potential of the radical, resulting in a less stable and less active form of GOase (Baron et al., 1994; Saysell et al., 1997). In this research, the optimization of residue W290 for D-glucose oxidation fostered a mutant with a phenylalanine residue substitution. The increased D-glucose activity suggested that the mutant's active center accessibility was improved and verified the role of Trp-290 in restricting substrate entry into active center. The

reduced interactions between D-galactose and Trp-290 for better access to the active center should account for the decreased D-galactose activity of M-RQW (1000 times) since k_{cat} of A3.E7 and M-RQW do not change that much (see Chapter IV).

Due to the limitation of screening methods, a single amino acid substitution, or a single base substitution, is usually preferred for the gene as large as GOase gene (2 Kb) in a directed evolution experiment. However, the number of accessible amino acids achieved with single base substitutions is only 5.6 on average and a large part of the protein sequence space cannot be explored (Miyazaki and Arnold, 1999). Three beneficial mutations were identified from the saturation mutagenesis experiments (Table 3.1). None of the mutations can be obtained from a single base substitution, illustrating the difficulty of obtaining D-glucose 6-oxidase activity in our random mutagenesis experiments.

Table 3.1. Mutations and the possible amino acid substitution by single base substitutions

Mutations	Accessible amino acid by a single base substitution
TGG→TTC (W290→F290)	R(AGG, CGG); G(GGG); S(TCG); L(TTG); C(TGC)
CGT→AAG (R330→K330)	S(AGT); G(GGT); C(TGT); H(CAT); P(CCT); L(CTT)
CAA→ACG (Q406→T406) CAA→AGC (Q406→S406)	K(AAA); E(GAA); H(CAC); P(CCA); R(CGA); L(CTA)

Our NMR results prove that mutant M-RQW has glucose 6-oxidase activity, an activity that has never been reported. The absence of this behavior in nature might indicate that the occurrence of glucose 6-oxidoreductase is a rare event in glucose oxidoreductase's evolution. The toxicity of glucose oxidoreductases might be a selective disadvantage, but it can be compromised by regulating the enzyme's production in fungi (Gancedo et al., 1966). The chemical reactivity of the glucose-dialdehyde might exist as a selective disadvantage as well; however, the presence of galactose oxidase, with the oxidation product of similar reactivity, reveals that the product toxicity is not a major hurdle.

Obtaining the rare function of the selective oxidation of the 6-hydroxyl of D-glucose was accomplished by uncoupling its biological fitness from its production using directed evolution. The new function was generated with three amino acid substitutions. Although the mutant M-RQW is a rather poor catalyst (Chapter IV), it proves that the generation of the selective oxidation of the 6-hydroxyl of D-glucose is possible, and nature should be able to easily produce a fully functional glucose 6-oxidase, as long as it endows a certain advantageous biological function in fungi. The absence of glucose 6-oxidase, therefore, indicates that sugar oxidoreductases may participate in certain metabolic functions, which can not be replaced by the activity of glucose 6-oxidase. It has been suggested that the product of fungal pyranose oxidase, 2-keto-D-glucose, is a key intermediate in a secondary metabolic pathway leading to the antibiotic cortalcerone (Baute et al., 1987). The oxidation products of sugar oxidoreductases may also participate

in secondary metabolic pathways and contribute to the chemical diversity of fungi.

4.Materials and Methods

4.1. Materials

All chemicals were reagent grade or better. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), D-galactose and horseradish peroxidase (HRP) were from Sigma (St. Louis, MO). *E. coli* strain BL21(DE3) was purchased from Novagen (Madison, WI) and the plasmid pGAO-36 containing GOase mutant A3.E7 gene was constructed and described previously(Sun et al., 2001). Restriction enzymes and ligase were obtained from Boehringer Mannheim (Indianapolis, IN), Life Technologies (Grand Island, NY) or New England Biolabs (Beverly, MA). A site-directed mutagenesis kit (Quick-Change) was purchased from Stratagene (La Jolla, CA).

4.2. Library Construction and Screening

The library Lib-RFQ, containing random mutations at three sites (R330, Q406 and F464), was constructed using a Quick-Change kit (Stratagene). Following standard protocol, the residue R330 was subjected to saturation mutagenesis first to generate Lib-R. Saturation mutagenesis at the residue F464 was performed to generate Lib-RF in the same way using the templates containing the mixture of the plasmids isolated from 100 clones from the library Lib-R. The library (Lib-RFQ) was constructed by performing saturation mutagenesis on

residue Q406 using the mixed plasmid templates of 2000 clones from the library Lib-RF. The beneficial mutants from Lib-RFQ were subjected to random mutagenesis, at the residue F195, to generate Lib-RFQF. The library Lib-W was constructed by employing saturation mutagenesis on the residues W290. The beneficial mutation identified from the library Lib-W was introduced into the mutants identified from the library Lib-RFQF. The primers used to construct the libraries were: 5′-GCTGACAAGCAAGGATTGTACNN(G/C)TCAGACAACCAC GCGTGG-3′ and 5′-CCACGCGTGGTTGTCTGA(G/C)NNGTACAATCCTTGCTT GTCAGC-3′ (for Lib-R), 5′-GGCCAACGACGTATTCCGNN(G/C)GAGGATTCA ACCCCG-3′ and 5′-CGGGGTTGAATCCTC(G/C)NNCGGAATTCCACGTCG TTGGCC-3′ (for Lib-RF), 5′-GGTTGTGGCGTCAGAGTC(G/C)NNATAATCTGG GGAGGCGGC-3′ and 5′-GCCGCCTCCCCAGATTATNN(G/C)GACTCTGAC GCCACAACC-3′ (for Lib-RFQ), 5′-GCGGTCTTCATATCGCAATGATGCA NN(G/C)GAAGGATCCCCTGG TGG-3′ and 5′-CAACCAGGGGATCCTTC (G/C)NNTGCATCATTGCGATATGAAGACCAC-3′ (for Lib-RFQF), 5′-CCATTG GAGGCTCCNN(G/C)AGCGGTGGCGTATTTGAGAAGAATGGCG-3′ and 5′-CGCCATTCTTCTCAAATACGCCACCGCT(G/C)NNGGAGCCTCCAATGG-3′ (for Lib-W). The PCR conditions were: 95 ^{o}C for 30 seconds, 18 cycles of 95 ^{o}C for 30 seconds, 55 ^{o}C for 9 minutes and 30 seconds, and 68 ^{o}C for 30 seconds. The amplified plasmid was subjected to *Dpn*I digestion for the degradation of wild-type plasmid. The libraries were purified with a Qiagen PCR purification kit before being transformed by electroporation into BL21(DE3). Transformed cells were spread on Luria-Bertani (LB) agar plates supplemented with 100 μg/ml

ampicillin and grown overnight at 30 ^{o}C. Single colonies were picked into deep-well palates (well depth 2.4 cm; volume 1ml; Beckton Dickinson Labware, Lincoln Park, NJ) and cells were grown for 12 h at 30 ^{o}C and 250 rpm, in 200 μl LB containing 100 μg/ml ampicillin (LB-Amp). The master plates were duplicated by transferring a 10 μl aliquot to a new deep-well plate containing 300 μl LB-Amp and grown for 12 h at 30 ^{o}C and 250 rpm. The cultures were then centrifuged for 5 minutes, at 5000 rpm, and the cell pellet was resuspended in 300 μl 100 mM of sodium phosphate (NaPi) buffer, pH 7.0, containing 0.8 mM of $CuSO_4$. Following addition of 1 mg/ml lysozyme (2 hours at RT), dilution (5-fold), and the addition of 0.125% (w/v) SDS (overnight at 4 ^{o}C), the GOase activity was assayed using a GOase-HRP coupled assay (Baron *et al.*, 1994). Aliquots of the cell extract were reacted with D-galactose and D-glucose at pH 7.0. The initial rate (for D-galactose activity assay), or endpoint (for D-glucose activity assay), of H_2O_2 formation was recorded by monitoring the HRP-catalyzed oxidation of ABTS at 405 nm on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). More than 10,000 clones were screened from the library Lib-RFQ, and 100 clones from both libraries Lib-W and Lib-RFQF. The clones with higher activity than the parent were isolated for further activity examination.

The selected clones were cultivated in LB medium, with ampicillin, at 20 ^{o}C overnight for plasmid purification. The purified plasmid was then transformed into BL21(DE3), and three single clones were picked for growth in a 3 ml LB medium with ampicillin (100 mg/L). After being cultivated for 12 hours at 30 ^{o}C, 15 μl of

culture was inoculated in 3 ml fresh LB medium with ampicillin. The inoculated culture was further incubated for 12 hours at 30 ^{o}C with shaking, and the cells were harvested by spinning down at 5,000 $\times$ g for 10 minutes. The cell pellet was resuspended by adding 1 ml of NaPi buffer (0.1 M, pH 7.0) with 0.4 mM of $CuSO_4$. After incubating with 1 mg/ml lysozyme at 30 ^{o}C for 1-2 hours, then with SDS (0.15%) at 4 ^{o}C for 2 hours, cell debris was spun down at 13,000 $\times$ g for 15 minutes. The activity of the supernatant for D-glucose and D-galactose was then evaluated by a HRP-ABTS coupled assay. The clones with higher activity than their parents towards D-glucose were further selected for sequencing.

4.3. Protein Purification and Characterization

E. coli culture was grown at 30 ^{o}C for 16 hours in LB medium (400 ml/2L flask) with 100 μg/ml of ampicillin. Cells were harvested with centrifugation, resuspended in 100 mM sodium phosphate buffer at pH 7.0, then disrupted by sonication. Cell debris was removed by centrifugation. The resulting supernatant was adjusted to 0.4 mM of $CuSO_4$, and stirred for approximately 2 hours at 4 ^{o}C. The samples were then loaded into a DEAE-cellulose column packed according to the manufacturer's instructions and eluted with 50 mM of sodium phosphate buffer at pH 7.0 and 4 ^{o}C. The active fractions were pooled and concentrated by a stirred ultrafiltration cell (Amicon Corp., Bevely, MA). The concentrated samples were dialyzed against 10 mM of sodium phosphate buffer at pH 7.3 overnight, and loaded into a cellulose phosphate column packed according to the manufacturer's instructions. After washing with 500 ml of sodium phosphate

buffer (10 mM, pH 7.3), the samples were further eluted by a linear gradient of 270 ml of 10 mM and 270 ml of 100 mM sodium phosphate buffer at pH 7.3. The active fractions were collected and concentrated in an Amicon ultrafiltration cell. The concentrated samples were dialyzed against 100 mM sodium phosphate buffer at pH 7.0 overnight prior to storage at –80 ^{o}C. The purified protein ran as a single band during SDS-PAGE (Novex, San Diego, CA). Protein concentrations were determined from the absorbance at 280 nm ($\in$ = 1.05 $\times$ 10^5 $M^{-1}cm^{-1}$), and corrected by a factor of 16/15 (Baron et al., 1994) for the mutant.

4.4. Oxidation of Methyl-β-D-Galacto-pyranoside

The oxidation of methyl-β-D-galacto-pyranoside with Sigma GOase was performed in a 5 ml solution of sodium phosphate buffer (50 mM, pH 7.0), 300 mM of substrate, 95 U of Sigma GOase, 700 U of catalase (Sigma), and 0.5 mM of copper ion. The oxidation of methyl-β-D-gluco-pyranoside with the mutant was performed in 2 ml of sodium phosphate (50 mM, pH 7.0), 200 mM of substrate, 1.8 U of mutant, 1700 U of catalase and 0.5 mM of copper ion. Both reactions were performed at room temperature, for 24 hours, with vigorous stirring.

4.5. TLC and NMR Analysis of Methyl-β-D-Galacto-pyranoside and Methyl-β-D-Gluco-pyranoside

TLC was used to monitor the oxidation reactions and isolate the reaction products. Chromatograms were developed with a chloroform-methanol (4:1) solvent and visualized with Bial's reagent (Sigma) on a TLC plate. A column

packed with silica gel (40 μ flash, Baker, Phillipsburg, NJ) was employed to isolate both reaction products eluted with chloroform-methanol (4:1). The samples were loaded into the column directly after the enzymes were removed using a centrifugal device (Millipore, Bedford, MA), and eluted with chloroform-methanol (4:1). The elutants were checked with TLC plates. The fractions with product were pooled and crystallized in a rotary evaporator. The crystallized samples were dissolved in D_2O and stored at 4 ^oC. Purpald reagent (Sigma) was also used to monitor the formation of aldehydes.

^1H 1–D and 2–D (COSY) NMR spectra and ^{13}C NMR spectra were recorded on a Varian-500 instrument. ^1H-NMR (500 MHz, D_2O, 300K) of methyl-β-D-6-aldo-gluco-pyranoside: δ=5.28 (d, 1H, H-1), 4.4 (d, 1H, H-6), 3.6 (s, 3H, -CH_3), 3.52 (m, 2H, H-3, H-4), 3.42 (m, 1H, H-2), 3.29 (m, 1H, H-5) — ^{13}C NMR (75 MHz, D_2O, 300K): δ=103.6 (C-1), 88.1 (C-6), 76.67 (C-5), 75.81 (C-3), 73.14 (C-2), 70.32 (C-4), 57.43 (CH_3).

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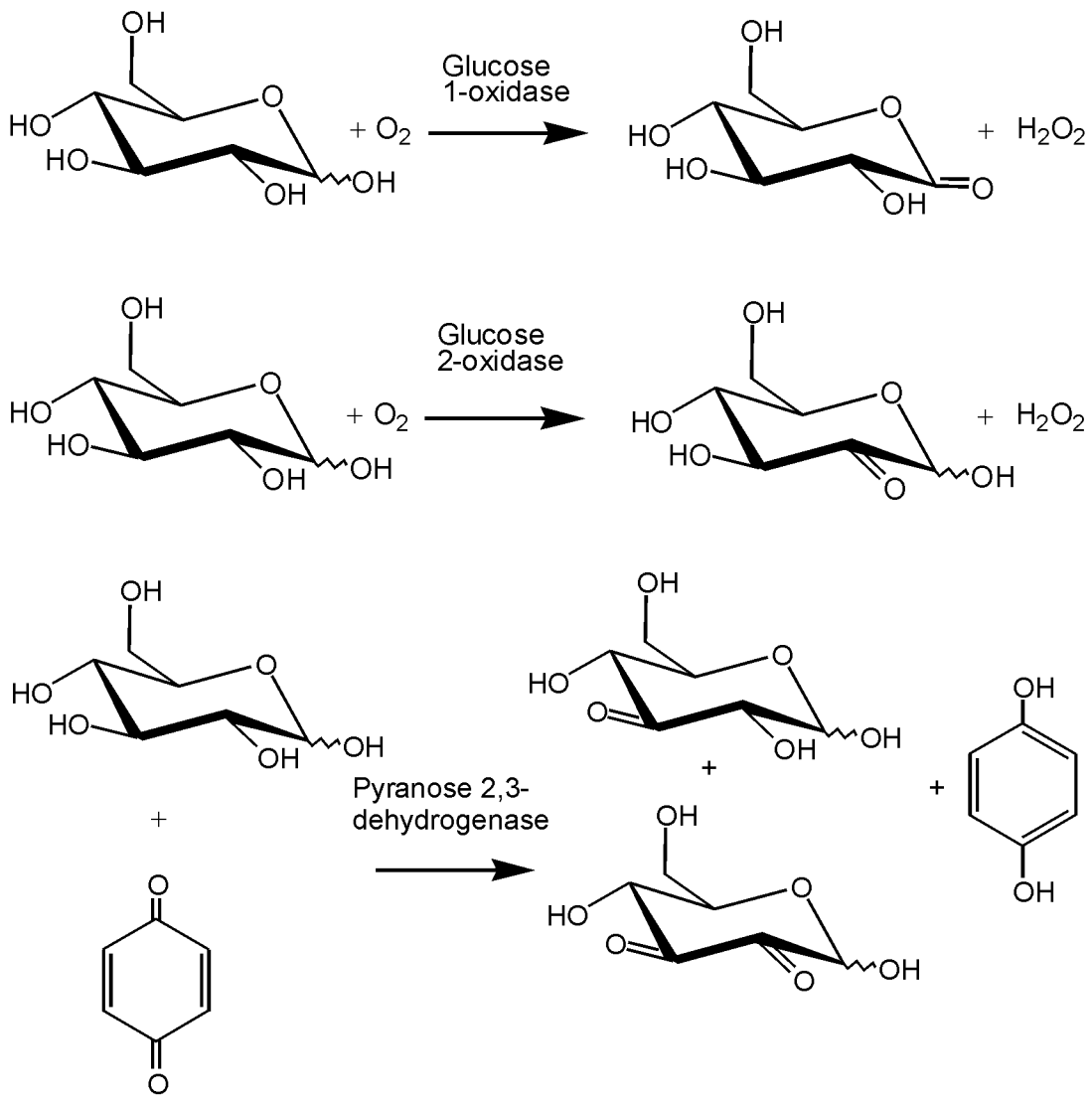
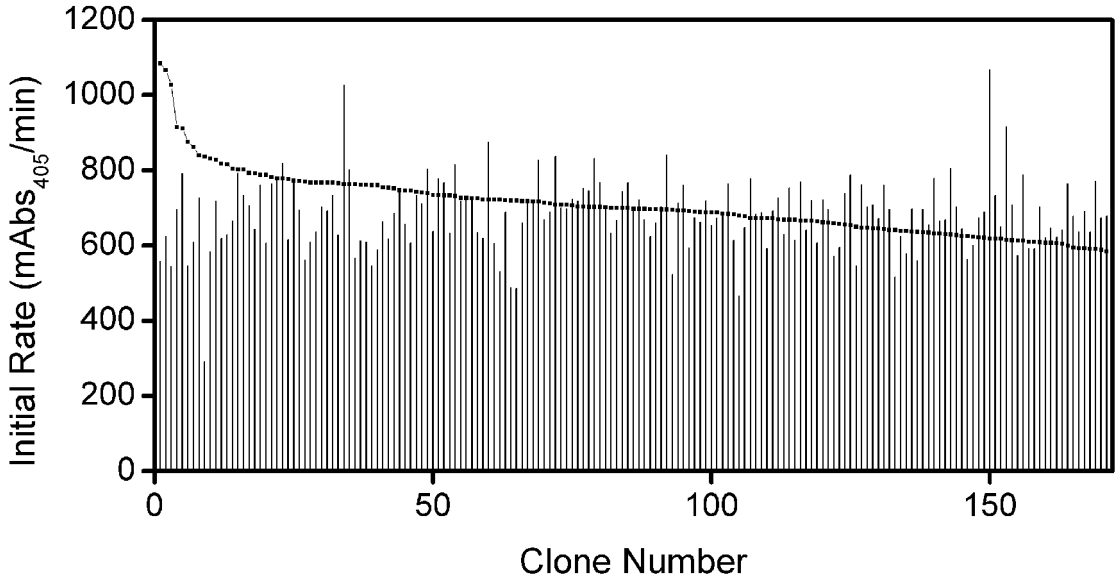
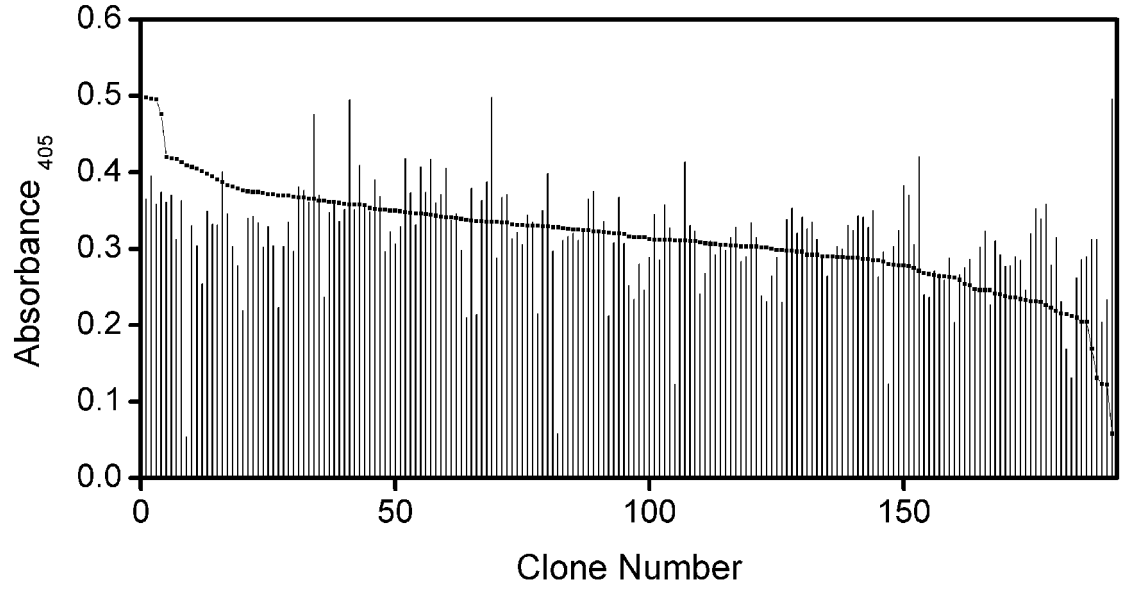


Figure 3.1. Reactions catalyzed by various glucose oxidoreductases.



A



B

Figure 3.2. Validation of the screening method. A3.E7 was used to evaluate the accuracy of the screening method for (A) D-galactose activity (B) D-glucose activity assay. The CVs for two activities are 14% and 21%, respectively.

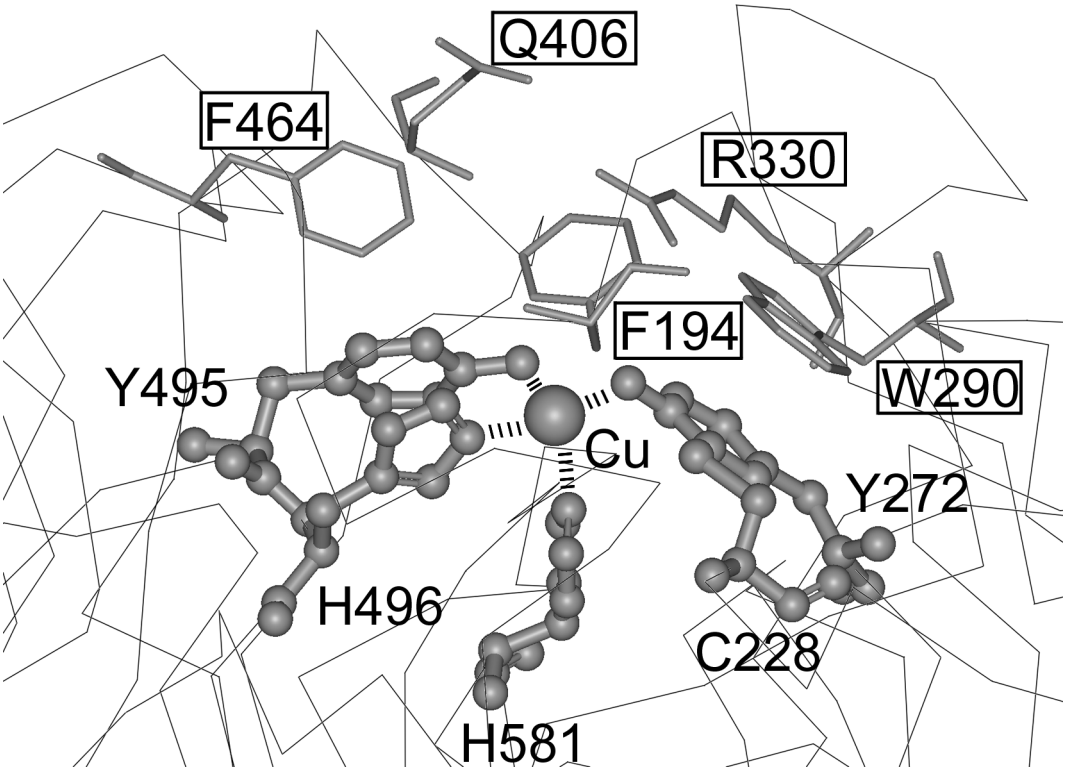


Figure 3.3. Residues R330, Q406, F464, F194, W290 targeted for saturation mutagenesis and key active site residues in GOase crystal structure.

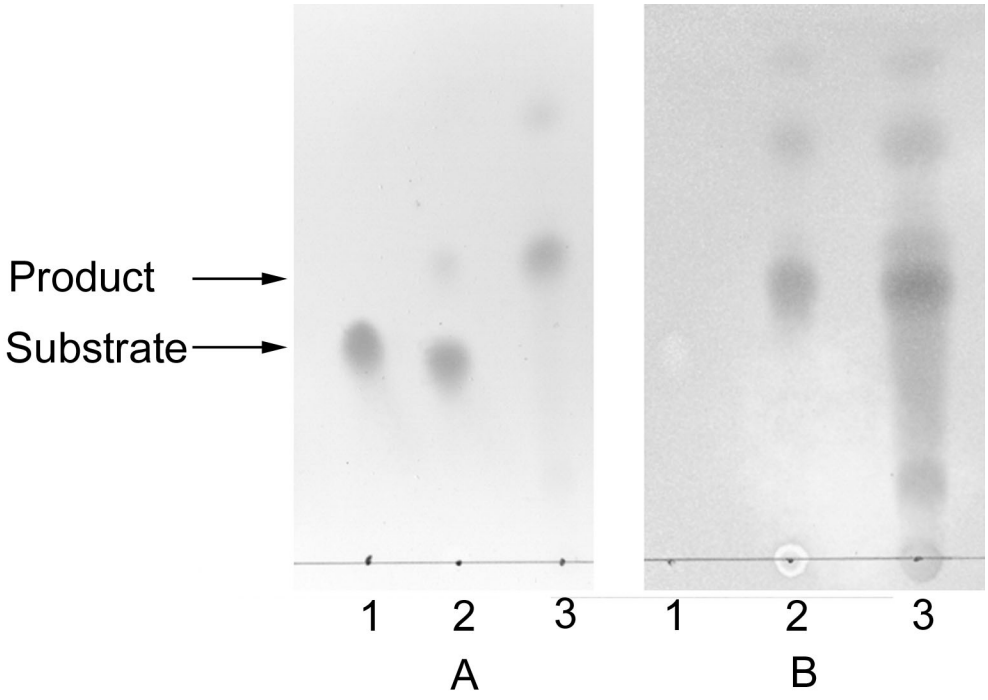


Figure 3.4. Thin layer chromatogram of reaction mixture of methyl-β-D-gluco-pyranoside oxidation by engineered GOase M-RQW. TLC plates were stained using Bail's reagent (A) and Purpald reagent (B). Line 1: substrate; Line 2: reaction mixture; Line 3: purified product;

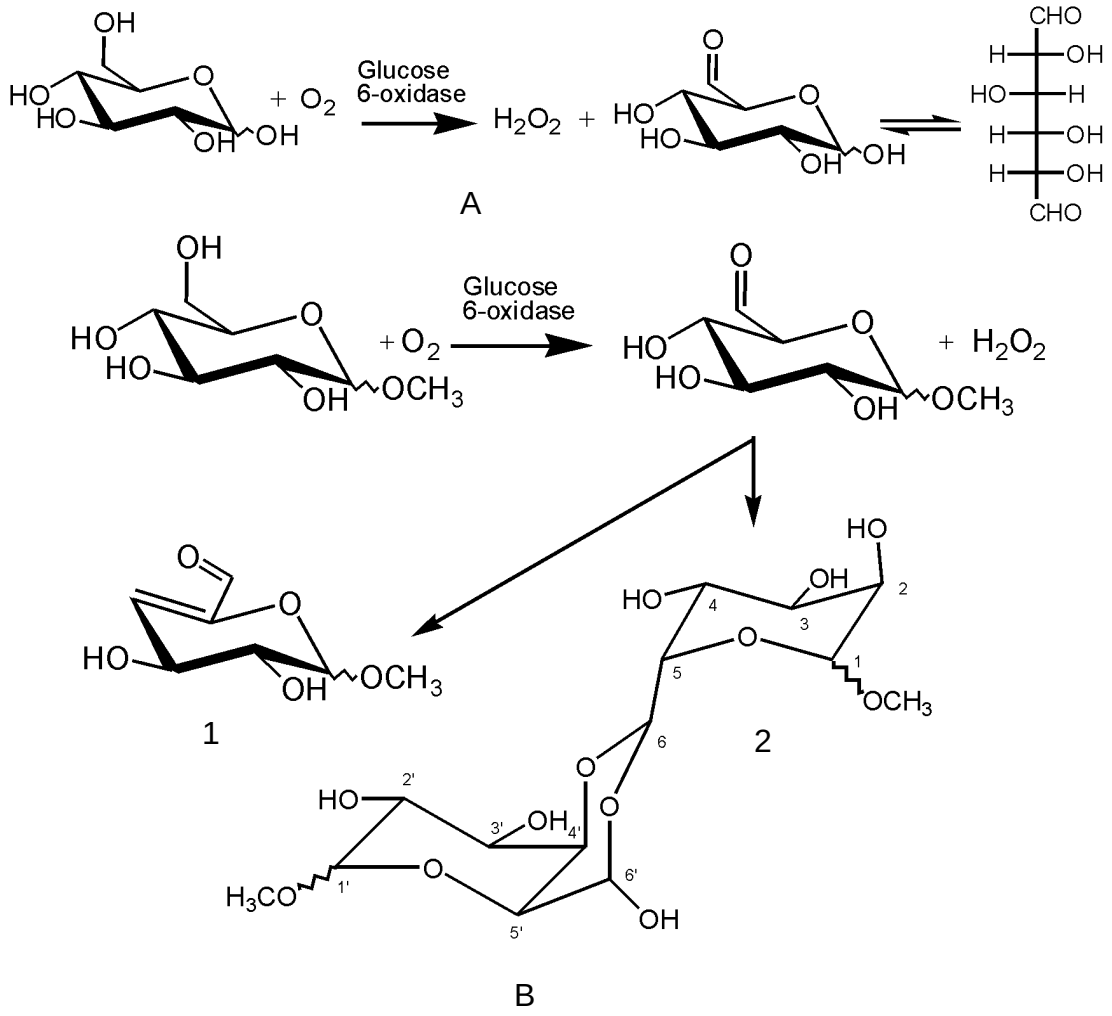
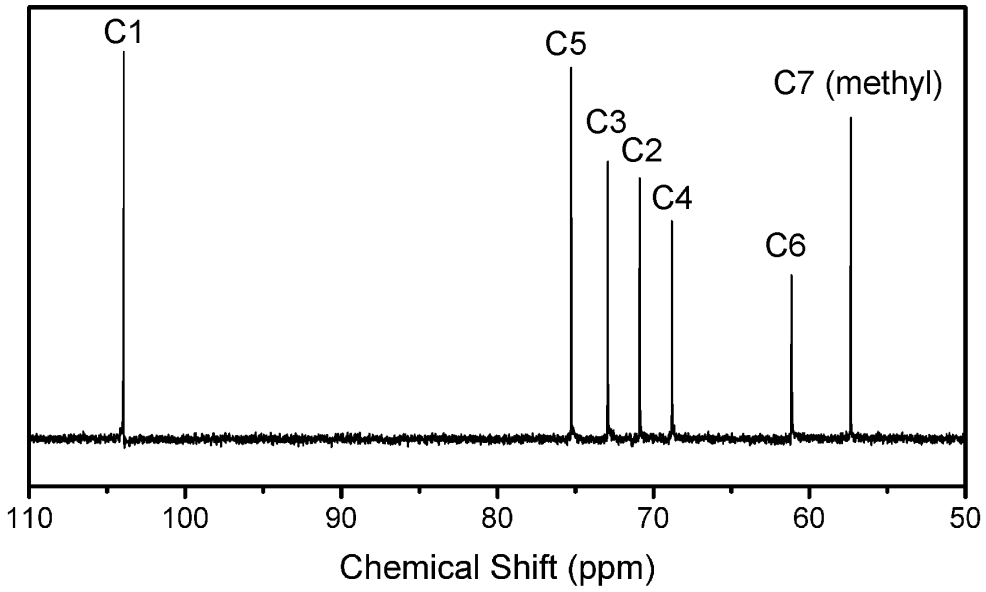


Figure 3.5. Oxidation reactions of D-glucose and methyl-D-gluco-pyranoside by mutant M-RQW, a glucose 6-oxidase. (A) Oxidation of D-glucose by M-RQW generates D-gluco-hexodialdo-1,5-pyranoside. (B) Oxidation of methyl-D-gluco-pyranoside by M-RQW produces methyl-β-D-6-aldo-gluco-pyranoside. This product can nonenzymatically form an α-β elemination product (1) and a dimer (2). Compound 2 can not be stained by Purple reagent, but the isolated product is always accompanied by small amounts of methyl-β-D-6-aldo-gluco-pyranoside, which can form color with Purple reagent.



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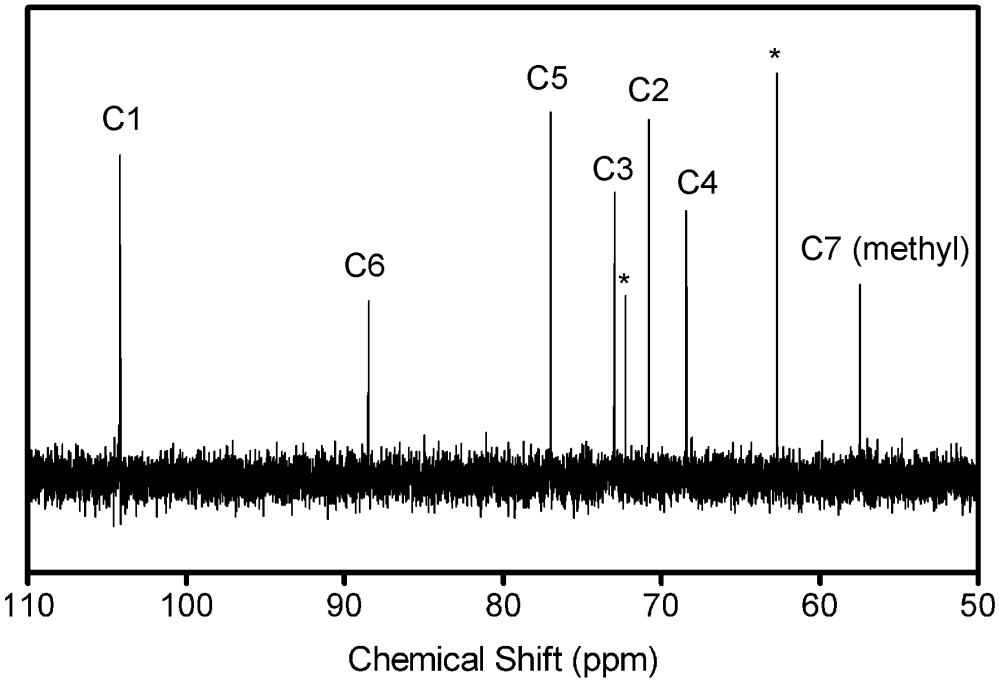
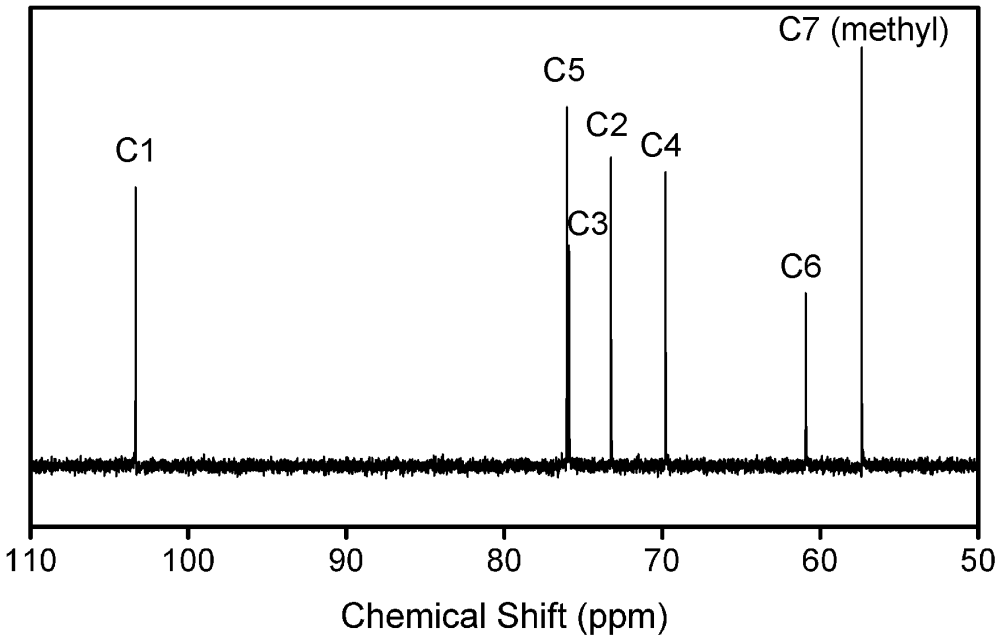
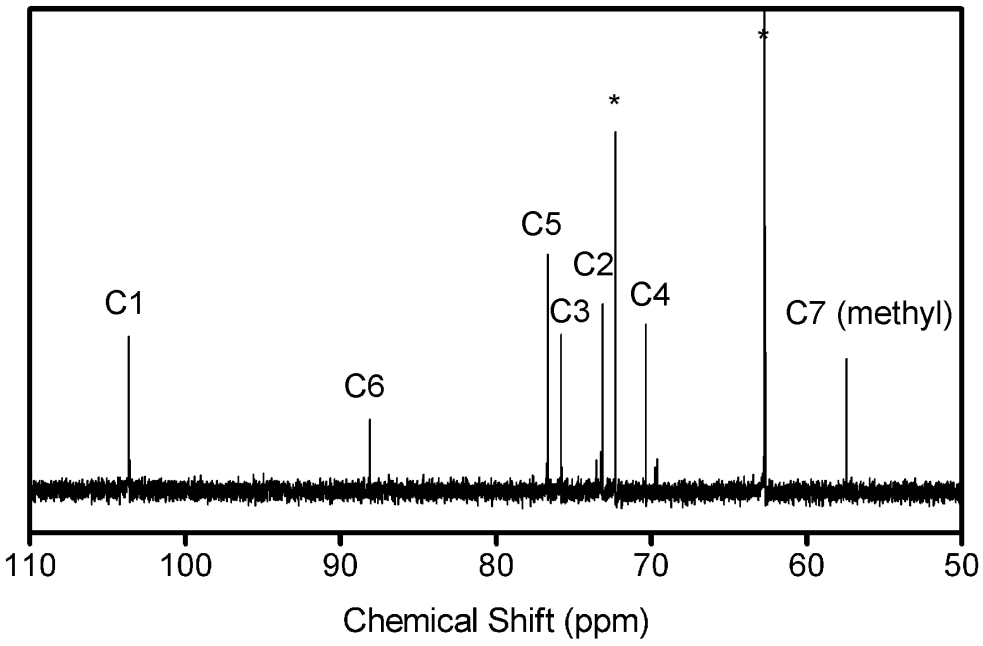


B

Figure 3.6. ^{13}C NMR of methyl-β-D-galacto-pyranoside and the oxidation product by the native GOase. The peaks indicated with stars are for glycerol. (A) NMR spectrum of the substrate (B) NMR spectrum of the product.



A



B

Figure 3.7. ^{13}C NMR of methyl-β-D-gluco-pyranoside and the oxidation product by the mutant M-RQW. The peaks indicated with stars are for glycerol. (A) NMR spectrum of the substrate (B) NMR spectrum of the product.

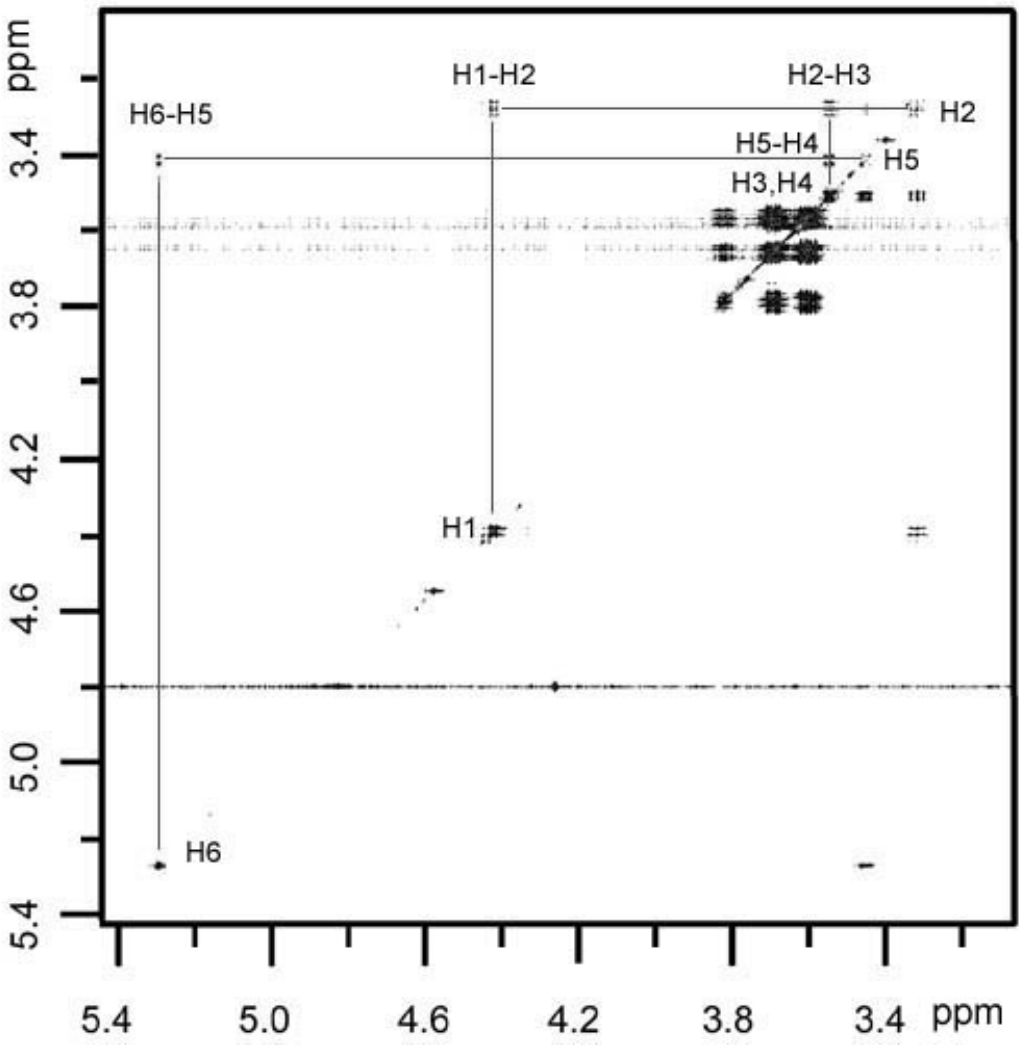


Figure 3.8. 2–D ^{1}H NMR of methyl-D-gluco-pyranoside oxidation product.

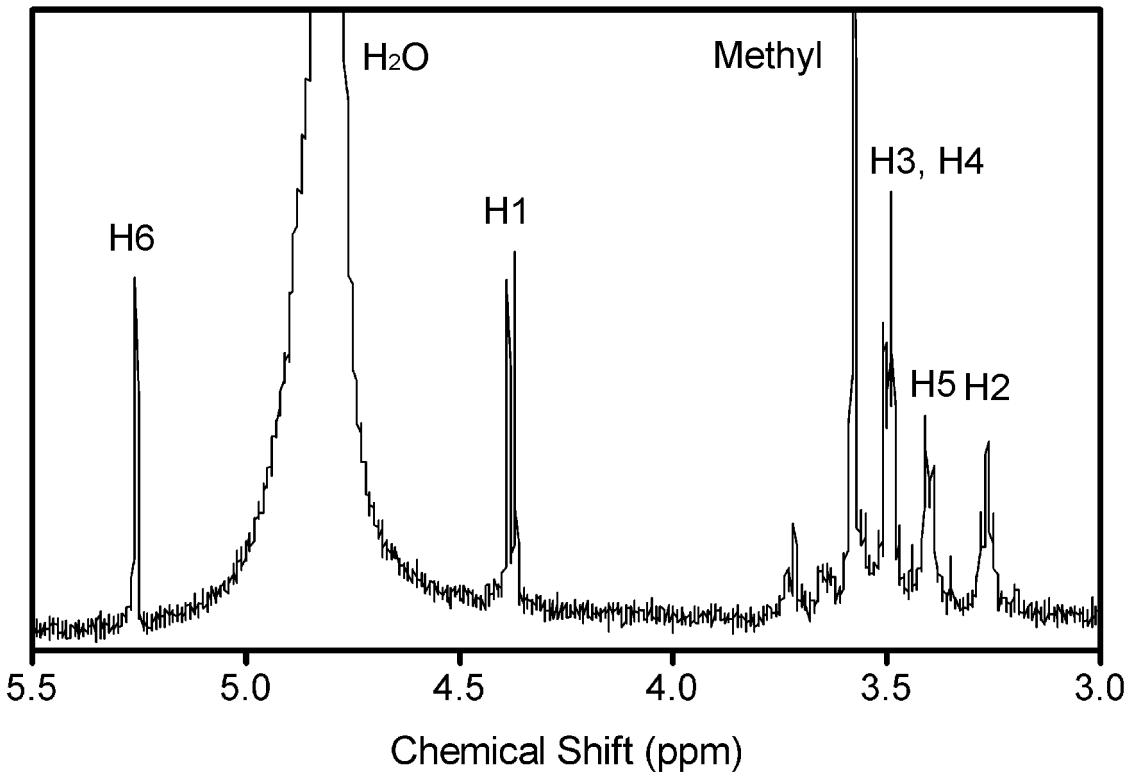


Figure 3.9. ^{1}H NMR of methyl-D-gluco-pyranoside oxidation product.

Chapter IV

Characterization of Mutant M-RQW With Glucose 6-Oxidase Activity

(Portions of this chapter have been published: Sun, L., Bulter, T., Alcalde, M., Petrounia I. P., and Arnold, F. H. (2002). *ChemBioChem*, 3, 781-783)

1. Introduction

Carbohydrate oxidoreductases are widely used in analytic chemistry, synthetic chemistry, and various fields of biotechnology. Glucose oxidase (β-D-glucose oxidase: oxygen-1-oxidoreductase, EC 1.1.3.4), a flavoprotein, has been successfully used in biosensors to detect a variety of carbohydrate compounds (Wilson and Turner, 1992). Galactose oxidase (GOase, D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9) also has been used to construct biosensors to measure carbohydrate compounds in food industry (Tkac et al., 1999; Vega et al., 1998). GOase has also been used in synthetic chemistry (Basu et al., 2000; Butler et al., 2001) and clinical research as well (Said et al., 1999). Pyranose oxidase (pyranose: oxygen 2-oxidoreductase; EC 1.1.3.10; synonym, glucose 2-oxidase) is mainly used in the synthesis of 2-keto-aldoses, an essential intermediate for sugar synthesis (Giffhorn, 2000; Giffhorn et al., 2000). An important lignin degradation enzyme, cellobiose dehydrogenase, has been proposed for application in the pulp and paper industry (Duarte et al., 1999).

We have applied saturation mutagenesis to introduce D-glucose 6-oxidase activity into galactose oxidase (GOase), an activity that has never been reported in nature or the laboratory. Although this activity is poor (1.6 U/mg) and might not be useful in synthetic chemistry, this enzyme (M-RQW) represents a promising starting material for further improvement of the activity. M-RQW can selectively oxidize the 6-hydroxyl group of D-glucose and its derivatives, which is expected to be useful in the food and paper industry by modifying the properties of

carbohydrate materials. In the pharmaceutical industry, this activity will be beneficial in the synthesis of carbohydrate antibiotics and vaccines. We are interested in the biochemical properties of this mutant, including its substrate specificity, kinetic efficiency and spectroscopic features. All of these properties are expected to be of use in the design of directed evolution experiments, the exploration of its potential applications, and the illustration of the reaction mechanism involved.

2. Results and Discussion

2.1. Protein Purification

The most convenient method to purify native GOase is to use Sepharose 6B as affinity scaffold in which native GOase can be selectively bind. This method has been successfully utilized to purify the poorly expressed recombinant GOase in *E. coli* (approximately 0.8 mg/L) (see Chapter II), and further purify partially pure commercial GOase samples (Hatton and Regoeczi, 1982). However, we found that this one-step affinity method was inadequate at purifying mutant M-RQW since mutant M-RQW does not bind to Sepharose 6B resin. It seems that the binding of the active center mainly contributes to the affinity between native GOase and Sepharose 6B, rather than its high basicity or the other D-galactose binding pocket in the first domain.

A two-step ion exchange purification method was employed to purify mutant M-RQW. This method has been used to purify GOase secreted from fungal species

Fusarium (Tressel and Kosman, 1980) or *Aspergillus* (Baron et al., 1994). Although M-RQW contains eight amino acid substitutions, and the negatively charged amino acids were introduced into highly basic GOase to compromise its pI (12) value, M-RQW can still be purified through this method, but with low yield and decreased efficiency (Table 4.1). Mutant M-RQW, mutant A3.E7, and wild-type GOase were purified with this method and the purified proteins were used for further characterization. The purified wild-type GOase is less active than the purified wild type using the Sepharose 6B affinity column. However, the catalytic efficiency of mutant A3.E7 purified by both methods is comparable. The less active wild-type GOase from this purification method probably was most likely generated by the inactivation that occurred during purification, which was much longer (around 3 days) than in the affinity purification method (1 day). Since mutant A3.E7 is more stable than wild-type GOase, the prolonged purification time inactivates wild-type GOase, but not mutant A3.E7.

2.2. Substrate Specificity of Wild-type GOase and Mutant M-RQW

The substrate specificity of mutant M-RQW is an important feature to investigate for its potential applications. A number of alcohols, including aliphatic alcohols, aromatic alcohols, monocarbohydrates, oligocarbohydrates, and polycarbohydrates, were used to characterize the substrate preference of mutant M-RQW and wild-type GOase. While the substrate specificity of wild-type and native GOase is similar, the profiles listed in Table 4.2 indicate that the substrate specificity of the mutant is significantly different from these forms of GOase.

Many substrates that were previously inaccessible by native GOase, including D-glucose, glucose derivatives, and aliphatic secondary alcohols, can be oxidized by the mutant M-RQW. Several primary alcohols, which have α-substitution with a carbonyl or aromatic group, are very active substrates with the mutant M-RQW.

D-galactose is still a fairly good substrate for M-RQW, which is 20 times more active on D-galactose than on D-glucose (Table 4.2). M-RQW has comparable activity on D-glucose and 2-deoxy-D-glucose. However, its activity on D-mannose, a 2-epimer of D-glucose, is 2000-fold less than on D-glucose, suggesting that the configuration of the 2-hydroxyl group is important for D-glucose activity. Maltose, amylose, cellulose, and starch can also be oxidized by mutant M-RQW, revealing the potential applications of M-RQW for modifying glucose polymers.

Mutant M-RQW shows very high activity on several aromatic alcohols (pyridylcarbinol) and α-keto alcohols. M-RQW's specific activity on its best substrate, 2-pyridylcarbinol, is in fact higher than the native enzyme on dihydroxylacetone (DHA or 1,3-dihydroxy-2-propanone), its best substrate. M-RQW's specific activity on DHA is also higher than native GOase on DHA, primarily due to more than 12 times decreased K_m (Table 4.3). These results indicate that the accessibility of the active center is augmented significantly in the mutant to create a more broadly specific oxidase, and the broadened substrate specificity dose not necessarily undermine the enzyme's catalytic activity.

2.3. Novel Activity Towards Secondary Alcohols

Mutant M-RQW shows activity towards several secondary alcohols, an activity absent in native GOase. Mutant M-RQW can oxidize several aliphatic secondary alcohols, and its reaction with 2-butanol was performed to identify the oxidation product. The reaction mixture was subject to GC-MS analysis without any pre-treatment. 2-Butanol and 2-butanone were used as references. The GC analysis indicated that there were two components in the reaction mixture, and their retention times were consistent with these of 2-butanol and 2-butanone (Figure 4.3). This suggested that the oxidation product was a ketone: 2-butanone. MS analysis of the two components revealed the same features as that of the two references, verifying the formation of 2-butanone (Figure 4.4).

The stereospecificity of mutant M-RQW was examined with the oxidation reaction of (*R*)-2-butanol and (*S*)-2-butanol. The specific activity towards (*R*)-2-butanol was approximately twice that of (*S*)-2-butanol, which indicates that the mutant M-RQW was slightly preferential to *R*-configuration when reacting with chiral aliphatic alcohols.

Several inorganic complexes were synthesized to mimic the catalytic activity of GOase (Chaudhuri et al., 1998; Chaudhuri et al., 1999; Wang et al., 1998). All these models have strict substrate specificity (only a few primary alcohols) with sluggish catalytic efficiency, and none of them can oxidize secondary alcohols (allylic or benzylic). The oxidation of secondary alcohols by mutant M-RQW

shows that GOase can still abstract the Cα-H with a reasonable rate while broadening its substrate specificity, indicating a flexible and robust active center configuration.

2.4. Organic Solvent Stability of M-RQW and Wild-type GOase

Some substrates we employed are not soluble in water and may inactivate M-RQW and wild-type GOase. The activity of M-RQW in DMSO aqueous solution was measured to compare the organic solvent stability of M-RQW and wild-type GOase. Both M-RQW and wild-type GOase show increased activity (more than 50%) when the concentrations of DMSO are as high as 30% (v/v) (Figure 4.1). When the concentrations of DMSO are higher than 30%, the activity of M-RQW and wild-type GOase decreased. However, when more HRP was added, the activity of both increased, indicating that the HRP was inactivated and M-RQW and wild-type GOase are still able to exhibit high activity in the high concentrations of DMSO solution. Both M-RQW and wild-type GOase show similar relative activity over a range of DMSO concentrations (Figure 4.1), revealing that their organic solvent resistances are identical.

2.5. Spectroscopic Characterization of the Radical of Mutant M-RQW

Spectroscopic research revealed that purified native GOase was a mixture of two oxidation states: the oxidized state (Cu^{2+}-$Tyr^{\bullet}$) and the semi-reduced state(Cu^{2+}-Tyr) (Whittaker and Whittaker, 1988). The oxidized state, which features the strong absorbance at 450 nm and 800 nm, can be converted from semi-reduced

state through inorganic oxidation. This EPR-silent oxidized state can also automatically decay to the semi-reduced state completely under air-free conditions and 30% maximally in the presence of oxygen, which involves a disulfide bond Cys515-Cys518 as the electron transient source (Wright and Sykes, 2001). The oxidized state of M-RQW decayed rapidly (in 1 hour) at room temperature showing that the M-RQW radical was less stable than its parent and the native GOase (stable for several hours). The mutant M-RQW still can be oxidized by the mild oxidant sodium ferricyanide (redox potential: 410 mV) which suggests that its redox potential is close to the potential of native GOase (400 mV) (Figure 4.2). The redox potential of a GOase mutant with amino acid substitution W290H is 730 mV. This mutant also displays a decreased catalytic efficiency (1000-fold less) and less stable radical (20 minutes) (Saysell et al., 1997). The redox potential of M-RQW does not change significantly (close to 400 mV), however, the radical stability changes from several hours to 1 hour, indicating that the radical stability in GOase is not only affected by electrochemical properties, but also is influenced by structural factors.

3. Materials and Methods

3.1. Protein Purification and Characterization

E. coli cultivation and the cell disruption were performed as previously discussed (see Chapter II). The samples were then loaded onto a DEAE-cellulose column and eluted with 50 mM NaPi, pH 7.0 at 4 ^{o}C. The active fractions were pooled and concentrated in a stirred ultrafiltration cell (Amicon Corp., Beverly, MA). The

concentrated samples were dialyzed against 10 mM of NaPi at pH 7.3 overnight, and loaded into a cellulose phosphate column. After washing with 500 ml of NaPi (10 mM, pH 7.3), the samples were further eluted with a linear gradient of 270 ml of 10 mM and 270 ml of 100 mM NaPi at pH 7.3. The active fractions were collected and concentrated by ultrafiltration. The concentrated samples were dialyzed against 100 mM NaPi at pH 7.0 overnight prior to storage at –80 ^{o}C. The purified protein ran as a single band during SDS-PAGE (Novex, San Diego, CA). Protein concentrations were determined from the absorbance at 280 nm ($\in$ = 1.05×10^{5} $M^{-1} \bullet cm^{-1}$) and corrected by a factor of 16/15 for the M-RQW.

3.2. Analysis of 2-Butanol Oxidation Reaction

2-Butanol oxidation by M-RQW was performed at room temperature in 100 μl of NaPi (50 mM, pH 7.0) containing 50 mM of substrate, 0.5 mM of copper ion, 0.9 U of mutant GOase, and 150 U of catalase.

GC-MS analysis of the 2-butanol oxidation reaction was performed on an HP 6890 series GC system with HP 5973 mass selective detector and Rtx-1 column (60 m $\times$ 0.3 mm $\times$ 5 μm, Restek, Bellefontaine, PA). Helium was used as the carrier gas with the flow rate 1 ml/min. The mass spectrometer was operated in the scan mode (8.17 scans/sec) for the mass range between 40 and 200 amu. The GC was temperature programmed as follows: initial temperature 70 ^{o}C, then at 5 ^{o}C/min to 100 ^{o}C, and at 20 ^{o}C/min to 200 ^{o}C. The inlet and transfer line temperatures were 200 ^{o}C.

3.3. Characterizations of M-RQW

The activity of M-RQW and native GOase (partial purified) on various substrates was measured in 1 ml of NaPi buffer (100 mM, pH 7.0) using an ABTS-HRP coupled assay, at room temperature. The activity of M-RQW on starch and α-cellulose (both in saturated solution) was determined in 1.5 ml of NaPi buffer (100 mM, pH 7.0) at room temperature with stirring for the end point assay. The initial rate was recorded by a Shimadzu (Columbia, MD) UV-Vis spectrophotometer at 420 nm (ε=42.3 $\times$ 10^3 $M.cm^{-1}$). The organic solvent stability of wild-type GOase and M-RQW was measured at room temperature mixed with 5 mM of D-galactose, 0.7 U of HRP, and 0.133 mg of ABTS in 1 ml of NaPi buffer with various amounts of DMSO. One unit of activity is the amount of enzyme that is able to produce 1 μmol of product per minute under the reaction conditions.

UV-Vis spectra of mutant M-RQW were recorded from 320 to 900 nm. Oxidation of GOase was performed in 100 of mM NaPi at pH 7.0, by incubating with 100 mM $K_3[Fe(CN)_6]$ for 10 min followed by removal of the oxidant on a Biogel column at 4 ^oC.

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Table 4.1. Purification table of the ion exchange column method (Mutant M-RQW)

	Crude	DEAE Cellulose	Cellulose Phosphate
Amount of protein (mg)	651	31	7.7
Specific Activity (U/mg)	0.078	1.9	3.3
Total Activity (U)	51	59	25
Purification Factor	1	24	42
Purification Efficiency (%)	100	115	49

Table 4.2. Substrate specificity of native GOase, wild-type GOase and M-RQW

Substrate	Native GOase Relative Activity	Wild-type GOase Relative Activity	Mutant M-RQW Relative Activity
D-Galactose	100	100	100 (8.2 U/mg at 100 mM)**
D-Glucose	0	0	4.8 (1.6 U/mg at 420 mM)
2-Deoxy- D-Glucose	0	0	3.9
Methyl-α- D-Gluco-pyranoside	0	0	2.4
Methyl-β- D-Gluco-pyranoside	0	0	3.3
D-Fucose	0	0	0.01
D-Fructose	0	0	22
Methyl-α- D-Manno-pyranoside	ND***	0	9.5×10^{-3}
Methyl-β- D-Manno-pyranoside	ND	0	8.0×10^{-3}
D-Mannose	0	0	2.0×10^{-3}
β- D-Lactose	26	48	19
Methyl-α- D-Xylo-pyranoside	0	0	0.86
Methyl-β- D-Arabino-pyranoside	0	0	0.26
Methyl-β- D-Galacto-pyranoside	123	128	98
2-Deoxy- D-Ribose	0	0	0
L-Galactose	0	0	1.5
Maltose	0	0	0.45

Raffinose	168	97	101
Melibiose	71	192	121
α-Amylose	0	0	4.6
Starch	0	0	Active
α-Cellulose	0	0	Active
2-Propene-1-ol	ND	0.25	2.5×10^3
2-Pyridylcarbinol	1.5	0.56	1.8×10^5
3-Pyridylcarbinol	31	5.4	3.6×10^4
4-Pyridylcarbinol	8.7	1.3	8.1×10^4
1,3-Dihydroxy-2-Propanone	ND	129	1.7×10^3
2-Propanol	ND	0	5.7
3-Buten-2-ol	ND	1.0×10^{-3}	6.9
3,3-Dimethyl-2-Butanol*	ND	0	5.6
2-Octanol*	ND	0	4.0
2-Butanol	ND	ND	3.0 (0.02 U/mg at 50 mM)

*In the saturated solution

** The specific activity of wild-type GOase on D-galactose is around 1000 times higher than that of M-RQW on D-galactose. The numbers in the bracket are the specific activity.

***ND: Not Determined

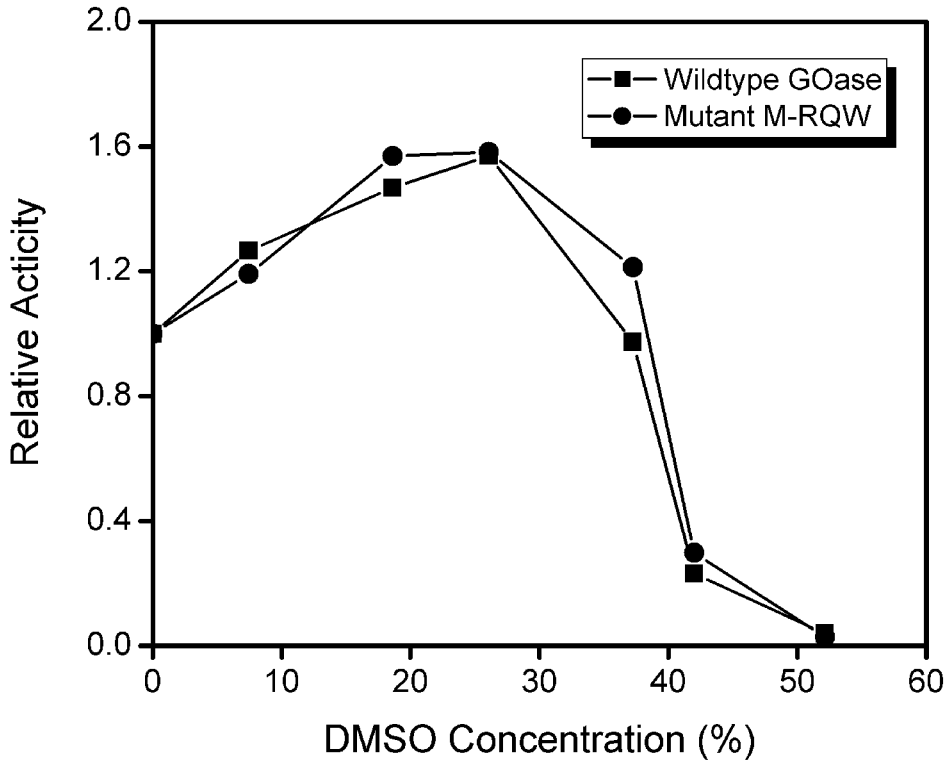

Figure 4.1. Organic solvent stability of M-RQW and wild-type GOase.

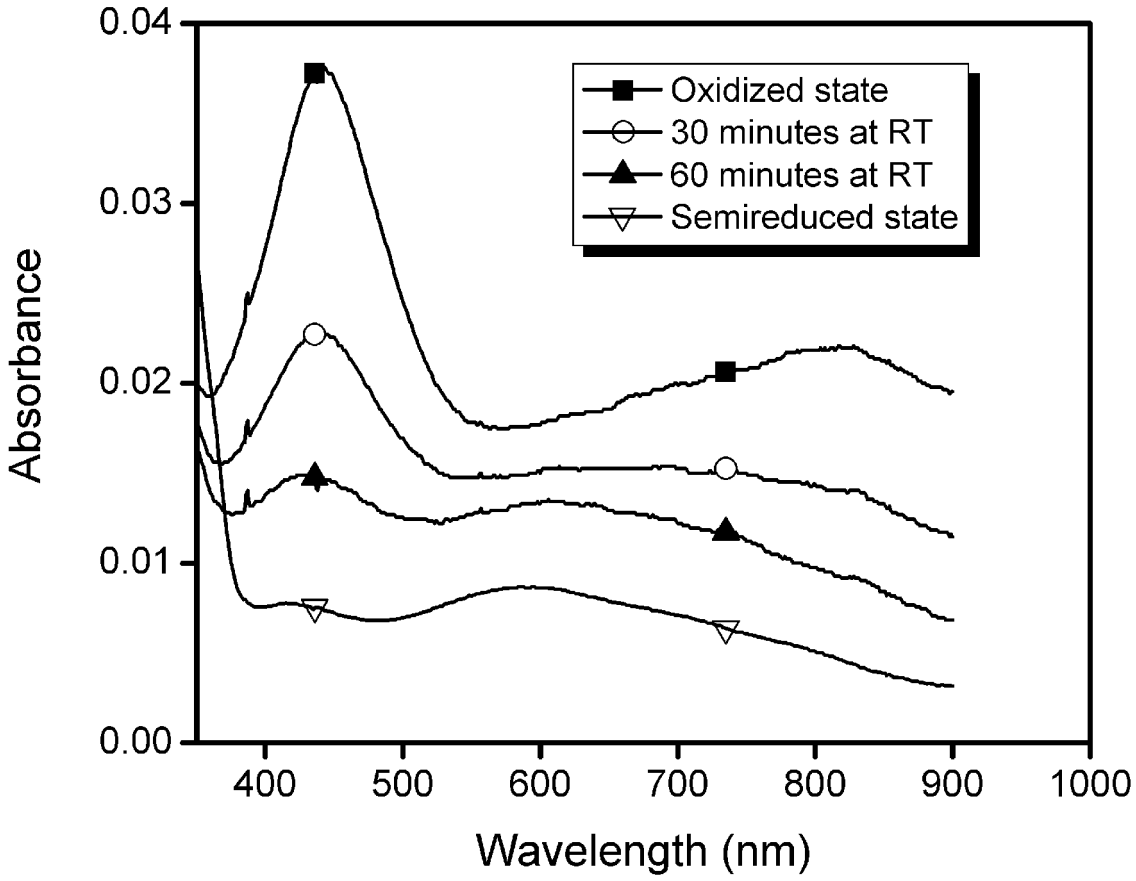

Figure 4.2. Optical absorption spectra of 7.7 μM M-RQW in the semireduced and oxidized states. Spectra of oxidized GOase were recorded immediately and at 30 minutes and 60 minutes following redox-activation by $K_3[Fe(CN)_6]$.

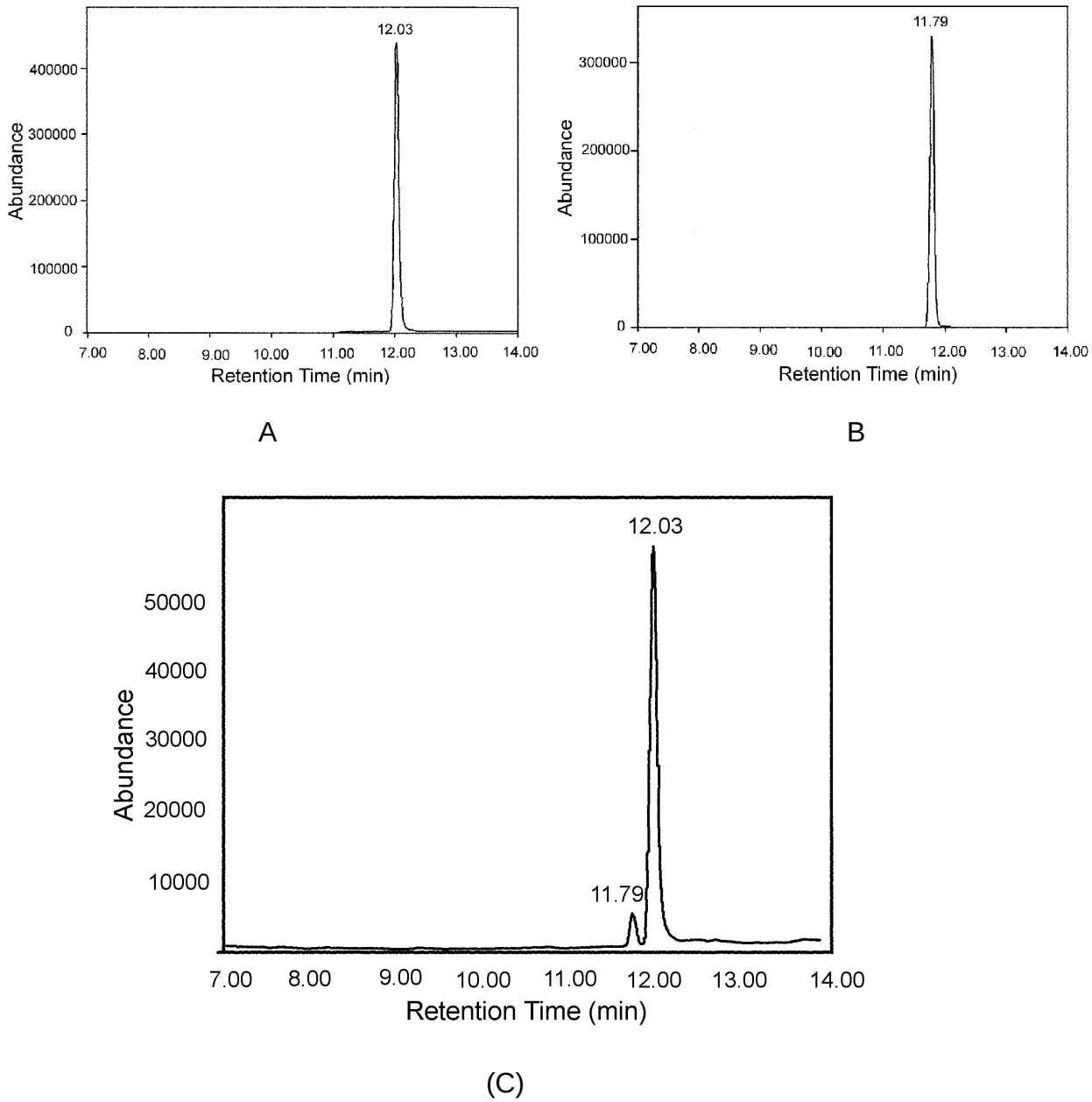

Figure 4.3. Gas chromatographic analysis of 2-butanol oxidation reaction catalyzed by M-RQW. (A) Gas chromatograms of 2-butanol. (B) Gas chromatograms of 2-butanone. (C) Gas chromatograms of the reaction mixture.

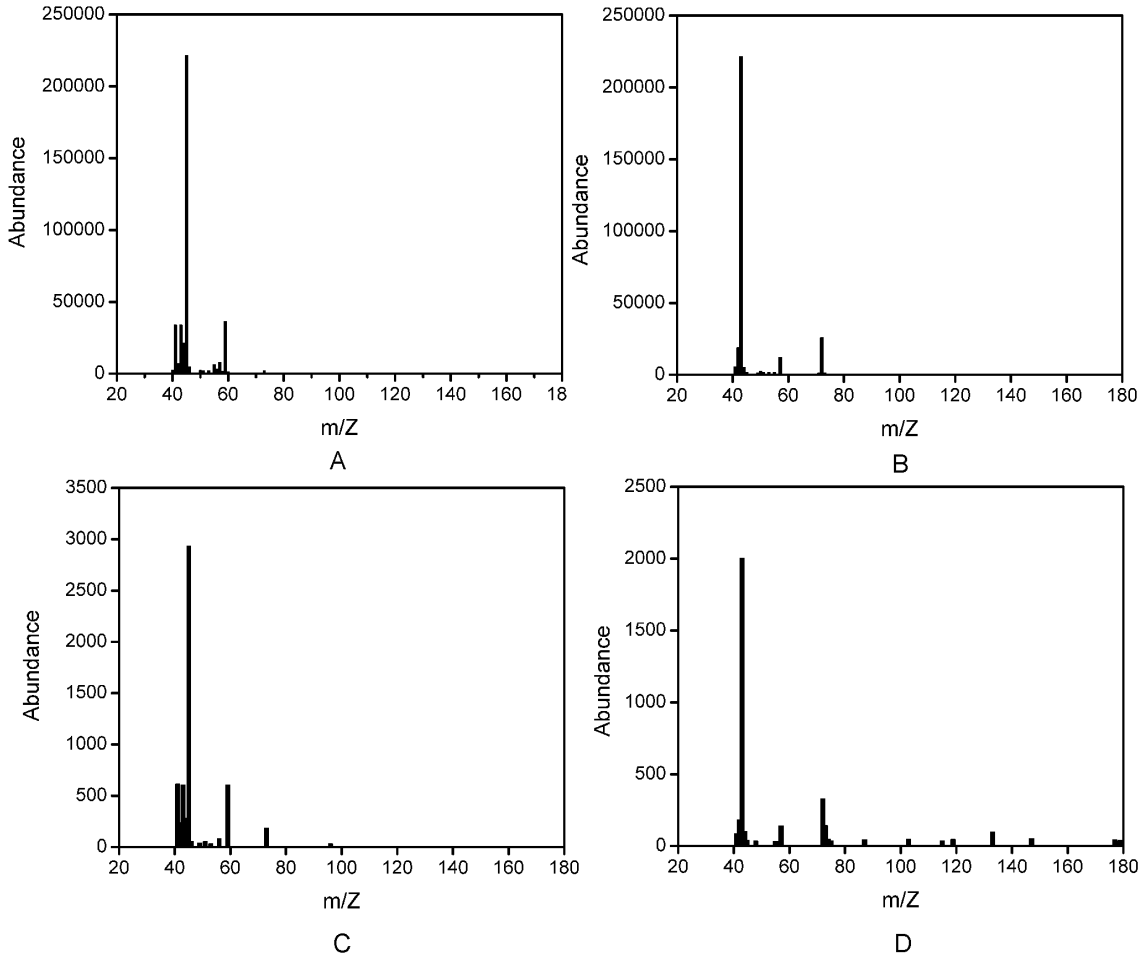

Figure 4.4. Mass spectra of 2-butanol reaction oxidized by M-RQW. (A) standard butanol. (B) standard butanone. (C) reaction mixture fraction with retention time of 12.03 minutes. (D) reaction mixture fraction with retention time of 11.79 minutes.

Conclusion

Random point mutagenesis combined with a high-throughput screen has been an important protein engineering method. However, it might not be an efficient tool to improve very weak enzymatic activities. A very sensitive assay method is required to monitor extremely low activities, and usually the reproducibility of the assay is compromised. However, structural information might be useful to increase such a low activity using saturation mutagenesis at a few selected sites as an alternative method. Combinatorial saturation mutagenesis utilizes structural information of enzymes to locate important amino acid residues to explore all the possible amino acid combinations of these residues. This optimization procedure might improve enzymatic activities significantly, circumventing the low reproducibility of screening methods. Unfortunately, enzyme structures do not always correctly reflect substrate binding information. Application of combinatorial saturation mutagenesis may not lead to desired functions in all cases.

Nature has generated a delicate tyrosyl radical in galactose oxidase to facilitate the oxidation of primary alcohols. GOase has to maintain a valid ligand configuration for its cupric ion, while it also has to preserve an efficient mechanism to generate the tyrosyl radical during maturation, stabilize it in an physiological environment, and regenerate it during a catalytic cycle. The unusually high conformational stability of GOase might be a mechanism to keep the tyrosyl radical away from environmental radical scavengers. Any mutational

efforts to engineer GOase have to maintain the mechanism for radical generation and stabilization, which is yet unknown.

Another tyrosyl radical enzyme, glyoxal oxidase, catalyzes the oxidation of aldehydes to carboxylic acids concomitant with the reduction of oxygen to hydrogen peroxide. Although glyoxal oxidase catalyzes different reactions from GOase, both enzymes have identical copper ligands and a cysteine-tyrosine cross-linked radical. However, the radical of glyoxal oxidase has much higher redox potential (640 mV vs. 410 mV) and is also much less stable (several hours vs. days), and is much less efficient (k_{cat}: less than 300 s^{-1} vs. 2999 s^{-1}) than that of GOase. It has been suggested that the distinct redox potentials for the radicals account for the different reactions catalyzed by two enzymes. Further mutagenic studies might be possible to tune the redox potential of the GOase radical to alter its catalytic reactions. On the other hand, it might be important to maintain the redox potential of GOase radical when altering its substrate specificity. The radical reaction mechanism of GOase adds an additional constraint for engineering studies.

Biogenesis of the thioether bond of GOase is a very interesting question. What oxidation chemistry is involved in this self-sufficient process? What role do the active center residues play to form the thioether bond? What is the mechanism of radical decay? The answers to these questions will enrich the chemistry and

mechanisms of radical enzymes, and will broaden our knowledge on how nature creates various mechanisms to control and regulate enzymatic reactions.