Energetics of Substrate Binding and Catalysis by Class 1 (Glycosylhydrolase Family 47) α-Mannosidases Involved in N-Glycan Processing and Endoplasmic Reticulum Quality Control*S

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Nascent glycoproteins are subject to quality control in the lumen of the endoplasmic reticulum (ER) where they can either be effectively folded with the aid of a collection of ER chaperones or they can be targeted for disposal in a process known as ER-associated degradation. Initiation of the ER disposal process involves selective trimming of N-glycans by ER α-mannosidase I and subsequent recognition by the ER degradation-enhancing α-mannosidase-like protein family of lectins, both members of glycosylhydrolase family 47. The kinetics and energetics of substrate binding and catalysis by members of this family were investigated here by the analysis of wild type and mutant forms of human ER α-mannosidase I. The contributions of several amino acid residues and an enzyme-associated Ca2+ ion to substrate binding and catalysis were demonstrated by a combination of surface plasmon resonance and enzyme kinetic analyses. One mutant, E330Q, shown previously to alter general acid function to substrate binding and catalysis were demonstrated by a combination of surface plasmon resonance and enzyme kinetic analyses. One mutant, E330Q, shown previously to alter general acid function within the catalytic site, resulted in an enzyme that possessed increased glycan binding affinity but compromised glycan hydrolysis. This mutant protein was used in a series of glycan binding studies with a library of mannose-containing ligands to examine the energetics of Man9GlcNAc2 substrate interactions. These studies provide a framework for understanding the nature of the unusual substrate interactions within the family 47 mannosidases involved in glycan maturation and ER-associated glycoprotein degradation.

As polypeptide chains are extruded through the endoplasmic reticulum (ER) membrane during co-translational translocation, they are commonly glycosylated on the amide side chains of Asn residues within the acceptor consensus sequon, Asn-X-(Ser/Thr) (1). Trimming of terminal glucose residues results in the formation of glycan structures that can act as ligands for the luminal ER lectin chaperones, calnexin and calreticulin (2, 3), which can aid in the folding of the nascent polypeptides in the lumen of the ER (2, 4). Glycoproteins that have slow folding kinetics continually re-engage the lectin chaperones either until folding is complete (2, 4, 5) or until the nascent glycoproteins acquire a target signal for disposal (6–8). For terminally misfolded glycoproteins, trimming of the oligosaccharide by the action of ER α-mannosidase I (ERManI) to generate a unique Man9GlcNAc2 isomer product (Fig. 1E) is the key rate-limiting initiation signal (9, 10) that ultimately leads to retrotranslocation of the polypeptide back into the cytoplasm for degradation by the proteasome in a process known as ER-associated degradation (ERAD) (11). Inhibition of ERManI can cause the accumulation of misfolded model glycoproteins in the ER lumen (12–20), and ERManI overexpression has been shown to accelerate the “disposal clock,” hastening the disposal of misfolded proteins and even early folding intermediates of wild type proteins (9). Thus, the efficiency of creating fully folded glycoproteins for transport from the ER is defined by a competition between the kinetics of conformational maturation versus the rate of acquiring the key glycan signal for glycoprotein disposal.

Many loss-of-function human genetic diseases result from delayed folding kinetics of potentially functional polypeptides, such as the ΔPhe638 mutant of cystic fibrosis transmembrane regulator (21), rather than generating terminally misfolded protein structures (22). Thus, treatment of many protein misfolding disorders could be achieved if pharmacological inhibition of the rate-determining steps for ERAD allowed sufficient time for completion of the protein folding process (7, 23).

ERManI is a member of a larger family of proteins, termed Class 1 mannosidases (24) (CAZy family 47 glycosylhydrolases (25–28)), involved in glycoprotein maturation and disposal. Two other subgroups within this family include a subfamily of hydroxylases in the Golgi complex and a subfamily of lectins in the ER. The Golgi α-1,2-mannosidases (termed IA (29), IB (30), and IC (31)) are essential for trimming high mannose N-glycans to the Man₃GlcNAc₂-Asn intermediate necessary for maturation into complex type structures on cell surface and secreted glycoproteins. In the ER, the EDEM subgroups of proteins apparently have no hydrolyase activity but act as lectins as a part of the ERAD disposal machinery (2, 32–38). The present models envisage recognition of the glycan structures by the EDEM proteins in a mode similar to substrate recognition during catalysis by the true hydrolases, followed by transfer to the Sec61 translocon pore, retrotranslocation into the cytosol, and proteasomal degradation (2). Thus, understanding how this family of enzymes and lectins accomplish their functions in recognition and catalysis will provide insights into the rate-
**FIG. 1.** Model for the structure and catalytic residues of human ERManI used in the mutagenesis studies described in this paper.

The end (A) and side (B) views of the human ERManI ribbon diagram (Protein Data Bank 1X9D (46)) display the (αααα) barrel structure with the N-glycan substrate (Man₉GlcNAc₂ substrate, stick representation from Protein Data Bank 1DL2 (46)) and the glycone residue in the –1 subsite.
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limiting decisions between glycoprotein maturation and disposal in the secretory pathway.

Class 1 α-mannosidases have been studied extensively with regard to enzyme kinetics, substrate specificity, structure, and mechanism (24, 39–46). The catalytic domains of enzymes from fungal and mammalian sources have similar (α2), barrel structures (41–45) that are plugged at one end by a β-hairpin, whereas the opposite end is composed of a broad cleft leading to the catalytic residues in the barrel core (Fig. 1, A and B). In all of the enzymes, a Ca\(^{2+}\) ion is bound at the apex of the β-hairpin in the core of the barrel where it is involved in direct interactions with two of the glycone hydroxyls during the catalytic cycle (Fig. 1, A–D) (42, 46). Co-complex structures have been examined between ERManI and the glycone mimic inhibitors, 1-deoxynojirimycin (dMNJ) and kifunensine (KitF) (Fig. 1F) (42), as well as a co-complex with an uncleaved thioglycoside pseudosubstrate (46), revealing a novel conformational itinerary during glycoside bond hydrolysis. Our recent studies suggest that the enzyme binds to the glycone residue in the −1 subsite in a high free energy S\(_1\) conformation (46), which allows the formation of a ring-flattened H\(_4\) transition state by a least motion conformational twist of the predisposed sugar ring, and produces an inverted enzymatic product in a C\(_4\) conformation. Novel general base (Glu\(^{330}\)) and general acid (Glu\(^{330}\) and Arg\(^{334}\) acting in a through-water protonation scheme) functions were identified through a combination of kinetic analyses and structure determination of the ERManI-thioglycoside co-complex (Fig. 1, C and D) (46). All of the known Class 1 mannosidase structures are essentially identical within the −1 and +1 subsites, suggesting that the catalytic mechanism for bond hydrolysis is conserved among all of the true hydrolyses.

Although all of the Class 1 mannosidases cleave Man-α,1,2-Man linkages, there are significant differences in branch specificities among the different family members (24). ERManI cleaves a single residue from the central branch of the Man\(_9\)GlcNAc\(_2\) substrate to produce a single Man\(_8\)GlcNAc\(_2\) isomer product (Fig. 1E) (39, 40, 47). In contrast, the Golgi subfamily of enzymes recognizes the other terminal Man-α,1,2-Man branches but instead cleaves the central branch with poor efficiency (48, 49). Thus, these enzymes have a mutually exclusive but complementary specificity for the complete cleavage of Man-α,1,2-Man linkages on high mannose glycans (41). Putative glycan enzymatic product co-complexes have been isolated for members of both the ER (43) and Golgi (41) subclasses of enzymes, demonstrating that differences in the cleft structures leading from the catalytic core residues confer unique glycan branch specificities for the different subfamily members.

In the studies described here, we have complemented and extended our recent work on the characterization of the ERManI catalytic mechanism (46) by examining for the first time the energetics of substrate binding and catalysis by a class 1 α-mannosidase. Kinetic and binding analyses have allowed us to dissect the energetic contributions of individual amino acid residues and the protein-bound Ca\(^{2+}\) ion during substrate binding and catalysis. Through the use of a general acid catalytic mutant that is compromised in hydrolysis, yet maintains substrate binding with similar characteristics to the wild type enzyme, we also mapped the contributions of individual residues in the Man\(_9\)GlcNAc\(_2\) substrate for their interactions with the extended enzyme glycan binding pocket. These studies revealed unanticipated roles for glycan interactions in the +1 subsites for facilitating catalysis and substrate specificity. The experimental strategy for the binding and kinetic analyses also provides a framework for further studies on the structural and energetic basis of substrate branch recognition and catalysis by other members of the class 1 (glycosyl)hydrolase family 47 α-mannosidases.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of Human ERManI—The mutagenesis, expression, and purification of the human ERManI catalytic domain has been described previously (42, 46). Briefly, the cDNA encoding the human ERManI catalytic domain in the pPICZαA vector (Invitrogen) was used to perform site-directed mutagenesis using the QuickChange\textsuperscript{TM} mutagenesis kit from Stratagene (La Jolla, CA). Plasmid constructs were then used to transform the Pichia pastoris strain X-33, and Zeocin-resistant colonies were screened for ERManI expression by performing Western blots using conditioned medium from induced cultures as described previously (42, 46). Mutant enzymes were expressed in 1-liter shake flask cultures by induction in BMM media, and the enzyme was purified from the conditioned media as described previously (42, 46).

Glycopeptide and Glycan Isolation—Man\(_9\)GlcNAc\(_2\) glycopeptides were isolated from crude soybean agglutinin by reduction, carboxymethylation, elution digestion, affinity chromatography using concanavalin A-Sepharose, and further purification by HPLC on a Cosmosil 118 column as described previously (46). Man\(_9\)GlcNAc\(_2\) was liberated from the peptide by peptide:N-glycosidase F digestion (50) and derivatized with pyridylamine (Man\(_9\)GlcNAc\(_2\)-PA) (48). Man\(_9\)GlcNAc\(_2\)-PA isomers, Man\(_9\)Glc\(_2\)NAc\(_2\)-PA, and Man\(_9\)Glc\(_2\)NAc\(_2\)-PA, were generated by digestion with either ERManI or Golgi ManIA and isolation by reverse phase HPLC as described (48).

Enzyme, Protein, and Carbohydrate Assays—The purified wild type and mutant enzymes were assayed for α,1,2-mannosidase activity using Man\(_9\)GlcNAc\(_2\)-PA as substrate as described previously (46). Briefly, the enzyme reactions (20 μl) were carried out in 96-well plates at 37 °C for the indicated times, stopped by the addition of 20 μl of 1.25% Tris-HCl, pH 7.6, and resolved and quantitated using a Hypersil APS-2 NH\(_2\)-HPLC column (48). One unit of enzyme activity is defined as the amount of enzyme that generates 1 μmol of Man\(_9\)GlcNAc\(_2\) from Man\(_9\)GlcNAc\(_2\) in 1 min at 37 °C. Protein concentration was determined using the BCA protein assay reagent (Pierce) as described by the manufacturer. Oligosaccharide concentrations were determined by phenol-sulfuric acid assays (51).

Kinetic Analysis—Initial rates (v) for the enzymes were determined at various substrate concentrations ranging from 10 to 300 μM. The catalytic coefficient (k\(_{cat}\)) and Michaelis constant (K\(_{m}\)) values were determined by fitting initial rates to a Michaelis-Menten function by nonlinear regression analysis using SigmaPlot (Jandel Scientific, San Francisco, CA).

from Protein Data Bank 1X9D (46) bound in the core of the barrel highlighted by the dark blue circle. The glycone residue in the −1 subsite (light blue circle, B) is in direct association with the protein-bound Ca\(^{2+}\) ion (blue spacefill). The residues examined in this study are shown in the stereo diagram (C) where the stick representation of the Man\(_9\)GlcNAc\(_2\) glycan substrate is shown in yellow, the Ca\(^{2+}\) ion is shown as a blue spacefill, and the relevant residues described in the text are shown as stick diagrams. Residues mutated in this study are shown in green. The Glu\(^{330}\) residue studied previously (46) is shown as a light blue stick figure. Water molecules coordinating the Ca\(^{2+}\) ion are indicated by small red spacefill structures with interactions with the Ca\(^{2+}\) ion indicated by cyan dotted lines. Interactions between the carbonyl oxygen and O-γ of Thr\(^{388}\) and the Ca\(^{2+}\) ion are also represented by cyan dotted lines. Proposed acid, base, and nucleophile trajectories as described previously (46) are illustrated with magenta dotted lines. Hydrogen bonds are shown as green dotted lines. A schematic diagram demonstrating the interactions between the N-glycan and the active site (D) employ a similar color scheme for hydrogen bonding, acid, base, and nucleophile trajectories and Ca\(^{2+}\) coordination as C, with the exception of the hydrophobic stacking between Phe\(_{23}\) and the C4–C5–C6 region of the −1 residue (black dotted lines). Residue numbering of amino acid side chains in the respective subsites is indicated in the figure. The residue nomenclature and linkages for the monosaccharides in the Man\(_9\)GlcNAc\(_2\)-Asn substrate are indicated (E), and the linkage cleaved by ERManI is also indicated. A similar monosaccharide nomenclature is used in C and D to label the respective residues in the glycan structure. Labeling of the enzyme subsites with negative (glycone in the −1 subsite) or positive (+1 and +2 subsite residues) numbers reflects their respective positions relative to the glycosidic bond being cleaved (64). Schematic structures of α-α-mannose and the corresponding inhibitors, dMNJ and KitF, are shown in F.
where to obtain a stock solution of 80 tions in each solution were confirmed by atomic absorption and used to
adjusted to pH 7.0 by addition of NaOH. All stock solutions were
free buffer (20 mM MES, pH 7.0, 150 mM NaCl). The calcium-free
analyses using 20 mM MES/NaOH, pH 7.0, 300 mM NaCl, and 200
and were used to calculate activation energies (Ea, the analyte, which did not dissociate from the chip surface even with extensive washing.
SPR data for each concentration of analyte were collected in duplica
globally fit to a 1:1 Langmuir binding algorithm model to
calculate the on-rate (kₐ), the off-rate (kₑ), and the equilibrium dissociation
constant (kₑ/kₒ = Kₒ) using the BIAevaluation 3.1 software (56).
Alternatively, the maximal equilibrium sensorgram values were used to
calculate the saturation binding constant and calculate values for the equilibrium dissociation constant (Kₒ) directly.
Kₒ values measured at different temperatures were used to calculate
thermodynamic parameters (56, 57) of binding using van’t Hoff equa
tion (Equation 5), (58) and Gibbs free energy change for binding (∆G)
was also calculated (Equation 6). The van’t Hoff equation allows the
calculation of thermodynamic parameters using the linear relationship of
y = ln(Kₒ) versus 1/T, which gives a slope of ∆H/R and an intercept of −∆S/R (58).
The effects of temperature on the association rates (kₐ) and dissociation
rates (kₑ) were independently determined using the Eyring equa
tion (Equation 7) (58).

\[ \ln K_o = \Delta H/R - \Delta S/R \]  
Equation 5

\[ k = (k_{3}T/h) \exp(-\Delta S/R) \exp(-\Delta H/R) \]  
Equation 7

 Similar to the van’t Hoff analysis, the Eyring equation allows thermo
dynamic parameters to be determined from measured kₐ and kₑ values at
different temperatures by a linear relationship of
y = Rln(hkₑ/hkₒT or y = Rln(hkₒ/hkₑT) versus x = 1/T, where the slope and the intercept of the
Eyring plots are −∆H and respectively, (58).

Isothermal Titration Calorimetry (ITC)—Calorimetry measurements
were performed with a 4200-ITC calorimeter (Calorimetry Sciences
Corp., Lindon, UT) as described (59, 60). Protein solutions for ITC
analysis were dialyzed overnight against buffer containing 20 mM MES,
pH 7.0, 150 mM NaCl, 5 mM CaCl₂ and 0.75 x 3-(1-pyridin-1-yl)-1-propane
sulfonate (NDSB201; Calbiochem) at 4 °C. The ligand solutions of Kif
dMNJ were prepared by diluting the compounds in the buffer used
for protein dialysis. Aliquots (5–10 µl) of the ligand solution (1–5 mM)
were automatically delivered into 1.3 ml of protein solution (1–2 mg/ml)
in the reaction cell. The calorimetry cell was allowed to return to
equilibrium for 4 min prior to the next injection. The data analysis
was performed using DataWork and BindWork software provided by manu
f acturer. Protein and glycan molecular structure figures were pre
pared using MacPymol (version 0.95)² to generate rasterized images.

RESULTS

Kinetic Analysis of ERManI Mutants—Mutations were gen
erated previously in five residues that were hypothesized to be
involved in catalysis by ERManI (46). These mutations tested the roles of putative general acid (E330Q and R334A) and
general base (E599Q and H524A) functions, as well as a resi
due that proved to play a critical role in providing hydrogen
bonding interactions with the mannose residue in the +1 subsite
(D463N) (Fig. 1, C and D). In the present study, we have
mutagenized two residues at the base of the +1 subsite (Phe⁶５⁹
and Thr⁶⁶⁸), one residue involved in interactions with several ≥
+1 residues (Arg⁶⁴¹), and one residue that interacts with both the
−1 and +2 subsite residues (Arg⁶⁹⁷) (Fig. 1, C and D), and
characterized their roles in catalysis and substrate binding.

Phe⁶⁵⁹ was shown previously to provide van der Waals inter
actions to the C4-C5-C6 region of the glycone in the −1 subsite
during catalysis (Fig. 1D) (42, 46). Thr⁶⁶⁸ is positioned at the
apex of the β-hairpin in the core of the (α₁)₁ barrel where it has
been shown to be the sole protein residue that directly coordi
nates the bound Ca²⁺ ion through both its O- and carbonyl oxygens
(Fig. 1D) (42, 46). The other four points of coordination of the Ca²⁺ ion to the enzyme are indirect through-water in

² W. L. Delano, Pymol Molecular Graphics System, available online at www.pymol.org.
Glycan and Inhibitor Binding Affinity Measurements to Human ERManI—In addition to kinetic analysis, the binding affinities of inhibitors and high mannose oligosaccharides to wild type and mutant forms of ERManI were also examined by SPR. Prior SPR studies with wild type and mutant forms of ERManI revealed significant alterations in the on-rates ($k_{\text{cat}}$) and off-rates ($k_{\text{doff}}$) of binding to dMNJ and Man$_9$GlcNAC$_2$-glycopeptide ligands (46). Correlations were made with the positions of the mutations and their impacts on ligand binding affinity. In the present study, we performed similar types of SPR studies with wild type ERManI in the presence and absence of Ca$^{2+}$, as well as testing the effects of the T688A, F659A, R461A, and R461L mutants on the binding of Man$_9$GlcNAC$_2$-glycopeptide, dMNJ, or Kif ligands (Fig. 2 and Table I). As described previously (46), the equilibrium dissociation constants ($K_D$) could be measured from a combination of the on-rates ($k_{\text{cat}}$) and off-rates ($k_{\text{doff}}$) determined by curve-fitting of the SPR sensograms, where $K_D = k_{\text{doff}}/k_{\text{cat}}$.

### Table I

| pH optimum$^a$ | $K_m$ (μM) | $k_{\text{cat}}$ (s$^{-1}$ × 10$^{-3}$) | $k_{\text{cat}}/K_m$ | $k_{\text{doff}}/K_m$ (wild type) |
|----------------|------------|-------------------------------------|---------------------|----------------------------------|
| Wild type$^b$  | 7.0        | 110 ± 8                             | 3,700 ± 110         | 33,000 ± 1,600                   | 100                                |
| E330Q$^c$      | 5.3        | 65 ± 1                              | 84 ± 3              | 1,200 ± 48                       | 3.5                                |
| T688A          | 6.5        | 15 ± 4                              | 60 ± 20             | 4,000 ± 1,300                    | 12                                 |
| F659A          | 6.8        | 120 ± 20                            | 30 ± 1              | 250 ± 6                          | 0.7                                |
| R461A          | 6.5        | 510 ± 130                           | 600 ± 100           | 1,200 ± 360                      | 3.6                                |
| R461L          | 6.5        | 330 ± 33                            | 210 ± 54            | 64 ± 4                           | 0.6                                |
| R597A          | 6.5        | 470 ± 38                            | 110 ± 6             | 230 ± 23                         | 0.7                                |

$^a$ Assay data were fit to generate a bell-shaped curve to yield a pH optimum with a standard error of <0.1 pH unit.

$^b$ Kinetic constants for wild type and E330Q mutant of ERManI were as reported previously (46) and are shown as a reference.

$^c$ The enzyme exhibits the ability to readily hydrolyze Man$_9$GlcNAC$_2$-PA to Man$_9$GlcNAC$_2$-PA.

**Corrections with carbonylate side chains in the core of the barrel.** Arg$^{461}$ interacts with several residues in the core of the Man$_9$GlcNAC$_2$-substrate (Fig. 1, D and E, residues M7, M4, and M3) and has been proposed to contribute to branch specificity for *Saccharomyces cerevisiae* ERManI (61). A Leu residue is found at the equivalent position in the Golgi subclass of enzymes. Previous mutagenesis studies generating the equivalent of an R461L mutant for *S. cerevisiae* ERManI (61) resulted in an enzyme that had a hybrid activity between the specificity of ERManI that cleaves only the central branch mannose residue and the Golgi mannosidases that cleave the remaining α,1,2-Man residues down to Man$_9$GlcNAC$_2$. Finally, Arg$^{597}$ appears to play a dual role in hydrogen bonding to the O-6 hydroxyl oxygen of the glycone in the −1 subsite via NH1 and the O-4 hydroxyl of the mannose in the +2 subsite via NH2. Each of the mutants (T688A, F659A, R461A, R461L, and R597A) was expressed in *P. pastoris* as a secreted catalytic domain, and the detailed enzyme kinetic parameters for hydrolysis of Man$_9$GlcNAC$_2$-PA were determined and are summarized in Table I. The pH optima for all of the mutant enzymes were slightly below the value for wild type ERManI (pH 6.5–6.8 versus 7.0 for wild type; Table I). The catalytic rates ($k_{\text{cat}}$) and the catalytic efficiencies ($k_{\text{cat}}/K_m$) of Man$_9$GlcNAC$_2$ cleavage for all of the mutants were significantly decreased, resulting in a range of $k_{\text{cat}}/K_m$ values that varied from 0.6 to 12% of wild type values. Surprisingly, the $K_m$ value for the T688A mutant was reduced 7.3-fold, whereas the Phe$^{597}$ mutant remained unaffected. In contrast, the $K_m$ values for the other mutants were all significantly increased by 3–5-fold.

For all of the mutants tested, the R461L mutant was unique in its ability to readily hydrolyze α,1,2-mannosidase residues from Man$_9$GlcNAC$_2$-PA to Man$_8$-GlcNAC$_2$-PA, as described previously for an equivalent mutant of *S. cerevisiae* ERManI (61) (data not shown). The 53-fold decrease in $k_{\text{cat}}$ and 3-fold increase in $K_m$ (Table I) indicated that although the amino acid substitution relaxed the specificity of the enzyme for glycan cleavage down to Man$_9$GlcNAC$_2$, the enzyme lost significant catalytic efficiency as a result of the mutation. In contrast, the R461A or R597A mutants were unable to cleave beyond Man$_9$GlcNAC$_2$. However, the catalytic rate of the R461A mutant was intermediate between the wild type enzyme and the R461L mutant. These data suggest that the removal of the Arg$^{461}$ side chain in the R461A mutant moderately compromised catalysis while retaining ERManI substrate specificity. In contrast the R461L mutant was altered in substrate specificity, although its catalytic efficiency was severely compromised.

The catalytic rates ($k_{\text{cat}}$) of wild type ERManI and the E330Q and T688A mutants were also obtained from initial rates measured at different temperatures but under optimal pH conditions for the respective enzymes (pH 7.1, 5.3, and 6.5 for wild type, E330Q, and T688A, respectively). The enzyme activities increased with temperature, and activation energies ($E_A$) were calculated from the slopes of the Arrhenius plots (supplemental Fig. 1A and supplemental Table I). The wild type enzyme and T688A mutant appear to have similar trends, with significant enthalpy and entropy contributions to the activation energy, whereas the E330Q mutant had a slightly reduced entropic contribution (supplemental Fig. 1A and supplemental Table I).

**Effect of the T688A Mutant of ERManI on Ca$^{2+}$ Ion Affinity and Enzyme Activity**—The role of the protein-bound Ca$^{2+}$ ion in catalysis by ERManI was determined by depleting wild type ERManI or the T688A mutant of bound Ca$^{2+}$ and then performing enzyme assays at defined Ca$^{2+}$ concentrations controlled by the presence of the divalent cation chelator EGTA. In the absence of any added Ca$^{2+}$, both proteins exhibited no detectable enzyme activity (supplemental Fig. 2). Addition of Ca$^{2+}$ resulted in a progressive appearance of enzyme activity, allowing the calculation of the Ca$^{2+}$ affinity for the enzyme, $K_{\text{Ca}}$, by curve-fitting. The $K_{\text{Ca}}$ values for the wild type enzyme (0.24 ± 0.02 μM) and the T688A mutant (0.15 ± 0.01 μM) were quite similar, yet the specific activity of the T688A mutant was generally 15-fold lower than the wild type enzyme at all Ca$^{2+}$ concentrations where activity could be detected. The observation that the T688A mutant is compromised in catalysis (reduced $k_{\text{cat}}$) but increased in substrate binding affinity (reduced $K_m$), while being essentially unaltered in Ca$^{2+}$ binding affinity, indicates that the mutation has a direct effect on catalysis rather than acting through a reduced affinity for binding and coordinating Ca$^{2+}$.
of a saturation curve for the equilibrium values of the binding sensorgrams (Fig. 2, inset plots) allowed an alternative means of determining the $K_D$ values (46).

Depletion of Ca$^{2+}$ from wild type ERManI resulted in a significantly slowed on-rate and off-rate for binding of the Man$_9$GlcNAc$_2$-glycopeptide ligand by SPR (Fig. 2), but resulted in only a 1.6-fold reduction in the equilibrium binding affinity for the glycan ligand (Table II). In binding studies where the kinetics for binding of the Man$_9$GlcNAc$_2$ ligand were too rapid for curve fitting, the equilibrium sensorgram values were used to plot a saturation curve (insets in the A and G plots) and calculate values for $K_D$. In the F659A mutant, binding of dMNJ was completely abolished as indicated by the absence of a deflection in the SPR sensorgram trace. The values for $k_a$, $k_d$, and $K_D$ based on the SPR studies are shown in Table II.

![Figure 2](image-url)

**Fig. 2.** SPR binding of Man$_9$GlcNAc$_2$, dMNJ, or Kif ligands to wild type (WT) and mutant ERManI. Wild type or mutant ERManI forms were immobilized on the SPR chip surface as described under “Materials and Methods,” and various concentrations of Man$_9$GlcNAc$_2$ (A, C, E, and G), dMNJ (B, D, F, and H) or Kif (I and J) ligands were tested for binding. The data were collected in duplicate, and representative SPR sensorgrams in the ligand concentration series are shown. If the on- and off-rates ($k_a$ and $k_d$, respectively) were sufficiently slow, curve fitting of the sensorgrams was performed using the 1:1 Langmuir binding algorithm model to determine the values for the equilibrium dissociation constants ($K_D = k_d/k_a$). In binding studies where the kinetics for binding of the Man$_9$GlcNAc$_2$ ligand were too rapid for curve fitting, the equilibrium sensorgram values were used to plot a saturation curve (insets in the A and G plots) and calculate values for $K_D$. In the F659A mutant, binding of dMNJ was completely abolished as indicated by the absence of a deflection in the SPR sensorgram trace. The values for $k_a$, $k_d$, and $K_D$ based on the SPR studies are shown in Table II.

In contrast to the effects of the T688A mutant at the base of the −1 subsite, mutation of Phe$^659$ (F659A), which provides van der Waals interactions with the −1 subsite residue, had a minimal effect on Man$_9$GlcNAc$_2$ binding (4-fold reduction in
The F659A mutant was reversible (Fig. 2), with measurable on- mutant. In contrast to the wild type enzyme, binding of Kif to enzyme (the binding of dMNJ was completely abolished for this mutant hybrid activity between ERManI and the Golgi subclass of interactions between Phe659 and the inhibitor at the base of the with Kif (data not shown). To test the role of the van der Waals binding (see supplemental Table II). The E330Q, D463N, and ITC confirmed the tight binding by the inhibitor, which was after extensive washing (Fig. 2). Inhibitor binding studies by detectable dissociation of the compound from the enzyme, even binding to wild type ERManI was examined by SPR. The equilibrium binding affinity for this inhibitor could only be estimated at <30 ns, because there was no detectable dissociation of the compound from the enzyme, even after extensive washing (Fig. 2). Inhibitor binding studies by ITC confirmed the tight binding by the inhibitor, which was highly exothermic and had a strongly favorable enthalpy of binding (see supplemental Table II). The E330Q, D463N, and T688A mutants also formed stable nondissociable complexes with Kif (data not shown). To test the role of the van der Waals interactions between Phe659 and the inhibitor at the base of the −1 subsite, we examined the binding of Kif to the F659A mutant. In contrast to the wild type enzyme, binding of Kif to the F659A mutant was reversible (Fig. 2), with measurable on- and off-rates and a KD of 1.45 μM (Table II).

Binding of Man9GlcNAc2 to the R461A and R597A mutants exhibited reduced on- and off-rates, but the KD values were similar to the wild type enzyme (data not shown). In contrast, the R461L mutant exhibited no detectable binding (KD > 1 mM) to Man9GlcNAc2 (data not shown), consistent with the poor catalytic efficiency of this mutant enzyme that has a hybrid activity between ERManI and the Golgi subclass of enzymes. Temperature and pH Dependence of Glycan Binding—In an effort to examine the temperature dependence of glycan binding to the wild type and mutant forms of ERManI, we performed a series of SPR binding studies with Man9GlcNAc2 as ligand at temperatures between 5 and 35 °C. The sensorgram responses for wild type ERManI, E330Q, and T688A (supplemental Fig. 3) are representative of the effects of temperature on the respective enzymes. Because of the fast on- and off-rates, binding to the wild type enzyme could only be measured at the equilibrium plateau values for the sensorsgrams, and below 15 °C there was little effect of increasing temperature (supplemental Fig. 3). At higher temperatures there was a progressive decrease in the sensorgram amplitude, presumably as a result of a combination of binding and hydrolysis of the ligand and subsequent release of the enzymatic product. For the T688A mutant, the off-rate progressively increased with increasing temperature, whereas the equilibrium sensorsgram values increased up to −20 °C and then progressively decreased (supplemental Fig. 3). We have interpreted these data to indicate that glycan binding was more prevalent than glycan hydrolysis at low temperature but that increased hydrolysis at higher temperature led to an increased off-rate and an inflection for the equilibrium sensorsgram values. In contrast, the off-rate for the E330Q mutant was considerably less influenced by increasing temperature, whereas the on-rate increased with temperature, leading to an increase in equilibrium binding (supplemental Fig. 3).

Finally, the binding of Kif to wild type ERManI was examined by SPR. The equilibrium binding affinity for this inhibitor could only be estimated at <30 ns, because there was no detectable dissociation of the compound from the enzyme, even after extensive washing (Fig. 2). Inhibitor binding studies by ITC confirmed the tight binding by the inhibitor, which was highly exothermic and had a strongly favorable enthalpy of binding (see supplemental Table II). The E330Q, D463N, and T688A mutants also formed stable nondissociable complexes with Kif (data not shown). To test the role of the van der Waals interactions between Phe659 and the inhibitor at the base of the −1 subsite, we examined the binding of Kif to the F659A mutant. In contrast to the wild type enzyme, binding of Kif to the F659A mutant was reversible (Fig. 2), with measurable on- and off-rates and a KD of 1.45 μM (Table II).

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To confirm the temperature dependence of enzyme activity under the conditions used for SPR analysis (pH 7.0) we compared the specific activities of the various enzyme forms at 10 and 37 °C at this pH. The wild type enzyme had a 30-fold increase in specific activity between 10 and 37 °C, whereas the T688A mutant increased 10-fold and the E330Q mutant increased only 2-fold over the same temperature range (data not shown). Because the E330Q mutant has a reduced pH optimum for catalysis (pH 5.3) relative to the wild type enzyme (pH 7.1) (46), we also tested the pH dependence of glycan binding by the E330Q mutant. At pH values ≥7.0, the E330Q mutant exhibits high affinity binding (KD < 1 μM) (Table III and supplemental Fig. 4), whereas at pH values below 7.0 both the on- and off-rates are altered to result in an enzyme with a 46-fold lower binding affinity at pH 5.0. We interpret the alterations in on- and off-rates and decrease in glycan affinity to reflect an increase in glycan hydrolysis under conditions closer to the pH optimum of the mutant enzyme. These data indicate that the E330Q mutant is an effective model for binding analyses under our conditions for SPR (pH 7.0, 10 °C) because, in contrast to wild type ERManI or the T688A mutant, there are minimal contributions of ligand hydrolysis.

**Table II**

Summary of the SPR binding affinity data for wild type and mutant forms of ERManI

| Ligand       | Protein            | Fitting type | kₐ | kᵦ | Kᵦ | Kᵦ/Kᵦ(wt) |
|--------------|--------------------|--------------|----|----|-----|------------|
| Man9GlcNAc2  | Wild type          | SS           | 48.6 ± 1.7 | 1   |
|              | E330Q              | 1:1          | 90.5 ± 16.3 | 1.6 |
|              | T688A              | 1:1          | 13.1 ± 3.5  | 0.94 ± 0.36 | 0.02 |
|              | F659A              | SS           | 194 ± 80.6  | 4.0 |
| dMNJ         | Wild type          | 1:1          | 2.94 ± 0.73 | 1   |
|              | E330Q              | 1:1          | 4.74 ± 1.47 | 1.61 ± 0.64 | 1   |
|              | T688A              | 1:1          | 11.95 ± 1.56 | 8.17 ± 1.68 | 5.1 |
|              | F659A              | ND           | ND |
| Kif          | Wild type          | 1:1          | >4.05       | <0.10 | <0.03 | 1 |
|              | F659A              | 1:1          | 7.38 ± 0.19 | 11 ± 0.1 | 1.45 ± 0.05 | >58 |

* 1:1 refers to a 1:1 nonlinear Langmuir fit of the binding data to derive the kₐ, kᵦ, and Kᵦ values from the kinetic SPR binding sensorsgrams as described under “Materials and Methods.” SS refers to a steady-state fit of the binding data from maximal equilibrium values of the SPR sensorsgrams as described under “Materials and Methods.” For examples of each type of data fitting, see Fig. 2.

* Binding analyses were performed at 10 °C.

* Immobilized surfaces were treated with EGTA overnight by continuous flow of running buffer containing 5 mM EGTA at 5 μl/min, and the binding analyses were performed in buffer containing 200 μM EGTA.

* ND, no detectable binding (KD > 10 μM).

* Binding analyses were performed at 25 °C.

Kₐ, see Table II), consistent with the lack of an effect of the mutation on the Kᵦ with the same glycan substrate. However, the binding of dMNJ was completely abolished for this mutant enzyme (KD > 10 μM; Fig. 2), consistent with 123-fold reduction in kᵦ (Table I). These data suggest that Phe659 plays a key role in glycan binding within the −1 subsite to promote catalysis.
Energetic Contributions of Glycan Binding to the E330Q Mutant of ERMa1—The E330Q mutant of ERMa1 was used as a model to investigate the contributions of various residues in a Man$_n$GlcNAc$_2$ oligosaccharide for binding to the mutant enzyme. Individual Man$_n$GlcNAc$_2$, Man$_n$GlcNAc$_3$, Man$_n$GlcNAc$_4$, and Man$_n$GlcNAc$_5$, glycans, as well as mannose disaccharides (Fig. 3A), were examined for their respective binding parameters (Table IV), and the resulting $K_D$ values were converted to their corresponding $\Delta G$ values for binding. The contributions of individual residues or groups of residues were subsequently calculated by a combination of difference calculations ($\Delta \Delta G$ values) using the rationale shown in Fig. 3B and Table V. These calculations indicate that binding of the glycone residue in the $-1$ subsite contributes only $\sim 21\%$ ($-1.68 \text{ kcal/mol}$) to the overall binding energy of a Man$_9$GlcNAc$_2$, ligand (Table V and Figs. 3C and 4), whereas the +1 subsite residue (M7 in Figs. 1E and 4) contributes $\sim 52\%$ ($4.2 \text{ kcal/mol}$) to the overall binding energy. Residues M9, M8, and M11 (Fig. 1E) together contribute $\sim 19\%$ ($1.53 \text{ kcal/mol}$) to the glycan binding energy, whereas the remainder of the glycan contributes $-0.42 \text{ kcal/mol}$ (6\%), and the peptide backbone contributions are negligible ($-0.19 \text{ kcal/mol}$) (Fig. 3C and Table V).

**DISCUSSION**

Class 1 (CAZy glycosylhydrolase family 47 (25–28)) $\alpha$-mannosidases play key and diverse roles in glycan maturation and disposal of misfolded glycoproteins in the ER (2, 9, 24, 32–36, 38). Prior studies have revealed that the overall protein fold for the catalytic domain of members of this family is an (a$a_1$)$_2$ barrel structure with a catalytic site in the core of the barrel (41–45). Co-complex structures between ERMa1 and dMNJ, Kif (42), or a mannobiery thiodisaccharide (46) revealed the barrel structure with a catalytic site in the core of the barrel (41–45). Co-complex structures between ERMa1 and dMNJ, Kif (42), or a mannobiery thiodisaccharide (46) revealed the conformational itinerary of the glycone during catalysis. Distortion of the glycone into a $\alpha_1^2$ conformation during substrate binding has been proposed to predispose the substrate for hydrolysis by a least motion conformational twist through a ring-flattened $\tilde{H}_4$ transition state producing an inverted enzymatic binding has been proposed to predispose the substrate for hydrolysis by a least motion conformational twist through a ring-flattened $\tilde{H}_4$ transition state producing an inverted enzymatic binding to the E330Q mutant of ERMa1. A series of SPR binding studies with the immobilized E330Q mutant enzyme was performed by using various ligand structures as indicated in A. In addition to Man$_n$GlcNAc$_2$-PA, Man$_n$GlcNAc$_2$-glycopeptide ligands, discrete isomers of Man$_n$GlcNAc$_2$-PA, Man$_n$GlcNAc$_3$-PA, and Man$_n$GlcNAc$_4$-PA, as well as Man$_n$1,2Man disaccharides, were tested. For each ligand, binding analyses were performed, and $K_D$ values were determined. Minimal on-rates and off-rates were both considerably altered at pH 5.0. Calculated values for $K_D$ indicated a progressive 40-fold lowering of glycan binding affinity between pH 8 and 5.

**Table III**

| pH   | $s^{-1}$ | $k_a$ | $k_d$ | $K_D$   |
|------|---------|-------|-------|---------|
| 8.0  | 33.8 ± 3.6 | 18.0 ± 0.8 | 0.536 ± 0.082 |
| 7.0  | 25.3 ± 0.1 | 11.8 ± 1.8 | 0.746 ± 0.079 |
| 6.0  | 10.8 ± 1.2 | 17.7 ± 0.1 | 1.64 ± 0.17  |
| 5.0  | 2.0 ± 0.2  | 41.5 ± 0.2  | 21.3 ± 2.35  |

**Fig. 3.** Strategy for determining the energetic contributions of respective Man$_n$GlcNAc$_2$ glycan residues for binding to the E330Q mutant of ERMa1. A series of SPR binding studies with the immobilized E330Q mutant enzyme was performed by using various ligand structures as indicated in A. In addition to Man$_n$GlcNAc$_2$-PA, Man$_n$GlcNAc$_2$-glycopeptide ligands, discrete isomers of Man$_n$GlcNAc$_2$-PA, Man$_n$GlcNAc$_3$-PA, and Man$_n$GlcNAc$_4$-PA, as well as Man$_n$1,2Man disaccharides, were tested. For each ligand, binding analyses were performed, and $K_D$ values were determined (Table IV). The resultant $K_D$ values were converted to values of $\Delta G$ (Table IV) by using the relationship $\Delta G = -RT \ln (1/K_D)$, and energetic contributions to glycan binding were then calculated by a series of $\Delta G$ difference calculations ($\Delta \Delta G$ calculations) as shown in the example calculation (B). The calculations for combinations of residues are summarized in Table V, and the data are graphically summarized in C. The glycone residue was found to contribute only 20.9\% to the overall glycan affinity, whereas the +1 subsite residue contributed 52.4\% to the glycan binding energy.

Two residues that were shown previously to play roles in wild type and mutant forms of ERMa1 by using various substrates and inhibitors.
performed by calculating the differences in free energies of binding for energetic contributions of individual or combinations of residues were detectable Man9GlcNAc2 binding (Fig. 4). Both the R461A and R597A had binding affinities that were comparable with 5-fold) (Fig. 4). The R461L mutation would eliminate the Leu residue at this position provides a positive role in binding, yet the latter mutant maintains the restricted subsite terminal glycan branches. However, the R461A mutant would be predicted to allow even greater flexibility for glycan binding to > +1 subsite residues (46, 61) were examined for their effects on catalysis and substrate binding. The R461L, R461A, and R597A mutants all resulted in reduced catalytic rates (varying 6-34-fold) and slightly increased $K_D$ values (3-5-fold) (Fig. 4). Both the R461A and R597A had binding affinities for dMNJ and Man$_9$GlcNAc$_2$ that were comparable with the wild type enzyme, whereas the R461L mutant had no detectable Man$_9$GlcNAc$_2$ binding ($K_D$ > 1 mM). Arg$^{461}$ has been proposed to play a major role in ERManI substrate binding and recognition (61), contrasting with a Leu at the equivalent position for the Golgi enzymes. Only the R461L mutant was found to readily catalyze the cleavage of Man$_9$GlcNAc$_2$-PA to Man$_n$GlcNAc$_2$-PA, similar to data reported for S. cerevisiae ERManI (61). Our working hypothesis is that Arg$^{461}$ contributes to high substrate binding affinity and substrate specificity only within the context of the overall geometry of the ERManI glycan binding cleft. The R461L mutation would eliminate specific hydrogen bonding interactions that confer glycan affinity and specificity as well as creating space at the core of the glycan-binding site for more flexible interactions with alternative terminal glycan branches. However, the R461A mutant would be predicted to allow even greater flexibility for glycan binding, yet the latter mutant maintains the restricted subsite specificity of wild type ERManI. These data suggest that the Leu residue at this position provides a positive role in broadening the substrate specificity, but within the inappropriate context of the ERManI glycan-binding cleft steric constraints preclude high affinity glycan interactions. In contrast, within the context of the active site clefts of the Golgi subfamily of enzymes, the corresponding Leu residue would be expected to confer high affinities of substrate binding and appropriate branch specificities for the latter enzymes.

Initial studies of inhibitor binding examined the energetics and kinetics of interactions by ITC and SPR. Both approaches indicated that binding of the inhibitors was highly exothermic, and the $K_D$ values from ITC were in close agreement with $K_D$ values from catalytic measurements and binding affinities by SPR (supplemental Table II). The major difference in structure between dMNJ and Kif is the fused five-membered ring in the latter compound (Fig. 1F) that causes Kif to be “pre-loaded” in the high free energy $C_4$ conformation prior to binding to the enzyme (46). The restricted conformation and the additional interactions between the ring and the Kif five-membered ring were proposed to account for the higher binding affinity of Kif in comparison to dMNJ (42). Surprisingly, binding studies by SPR indicated that the on-rate for binding to wild type ERManI was comparable between dMNJ and Kif, but the off-rate for Kif was considerably slower. Thus, the restricted conformation of the Kif six-membered ring did not accelerate binding of the inhibitor but significantly slowed dissociation. SPR binding studies between Kif and the F659A mutant, which should eliminate the van der Waals interactions with the C4-C5-C6 region of the ring-constrained inhibitor, significantly increased the off-rate and lowered the overall inhibitor binding affinity. Consistent with the lower binding affinity for Kif to the mutant enzyme, binding of dMNJ to the F659A mutant was not even detectable by SPR ($K_D$ > 10 mM). These data indicate that Phe$^{659}$ plays a critical role in stabilizing the glycone in the active site and that the van der Waals interactions with the ring-constrained inhibitor contributes to its slow dissociation rate from the active site. By extension, the greater ring flexibility of dMNJ or the substrate/product glycone residue associated with the wild type enzyme would be predicted to contribute an entropic component favoring dissociation from the active site. Consistent with a role for Phe$^{659}$ in inhibitor binding, the F659A mutant also caused a 123-fold reduction in $k_{cat}$, suggesting that interactions between Phe$^{659}$ and the glycone in the -1 subsite play a significant role in constraining the substrate into the $S_2$ conformation required for catalysis.

At the base of the -1 subsite a protein-bound Ca$^{2+}$ ion interacts directly with the glycone 2'- and 3'-hydroxyl residues. The ion is coordinated directly with the O-γ and carboxyl oxygens of Thr$^{688}$ and indirectly with four water molecules associated with Glu residues in the core of the -1 subsite. Two types of studies examined the roles of the Ca$^{2+}$ ion in glycan binding and catalysis. First, SPR binding studies on the Ca$^{2+}$-depleted enzyme indicated that the ion contributes to glycan on- and off-rates, but the enzyme had an almost identical equilibrium binding affinity for the Man$_9$GlcNAc$_2$ ligand as the enzyme containing bound Ca$^{2+}$. Binding of dMNJ was drastically reduced, with a predominant effect on reducing the on-rate of the inhibitor. Second, altering the Thr$^{688}$ side chain to

| Ligand | $k_a$ | $k_d$ | $K_D$ | $\Delta G^{298}$ |
|--------|------|------|------|----------------|
| Man$_9$GlcNAc$_2$-PA | 21.5 ± 1.5 | 15.2 ± 0.2 | 0.71 ± 0.04 | -7.96 ± 0.5 |
| Man$_9$GlcNAc$_2$-GP | 21.9 ± 4.9 | 11.1 ± 2.7 | 0.51 ± 0.17 | -8.15 ± 2.7 |
| Man$_9$GlcNAc$_2$-PA | 0.59 ± 0.01 | 14.9 ± 0.5 | 25.2 ± 0.4 | -5.95 ± 0.1 |
| Man$_9$GlcNAc$_2$-PA | 1.64 ± 0.06 | 31.9 ± 0.1 | 19.5 ± 0.8 | -6.10 ± 0.2 |
| Man$_9$GlcNAc$_2$-PA | 0.14 ± 0.02 | 29.9 ± 5.6 | 214 ± 16 | -4.75 ± 0.1 |
| Man$_9$2Man-0-CH$_3$ | 4.69 ± 0.21 | 163 ± 5.0 | 34.7 ± 2.5 | -5.77 ± 0.4 |
| Man$_9$2Man | 0.93 ± 0.25 | 26.1 ± 2.5 | 28.6 ± 5.0 | -5.88 ± 1.0 |

$\Delta G^{298}$ refers to purified glycopeptides from soybean agglutinin as ligands in the SPR binding studies.

**Table IV**

Summary of oligosaccharide binding interactions to the E330Q mutant of ERManI

**Table V**

Calculation of binding energy contributions for various glycan components to the E330Q mutant of ERManI establishing the +1 subsite residue as the major contributor to glycan binding.

Free energy calculations were generated by a strategy shown in Fig. 3B using a color convention for the residues in the high mannose ligands as indicated in the same figure. ΔΔG calculations to define the energetic contributions of individual or combinations of residues were performed by calculating the differences in free energies of binding for various glycan ligands (from Table IV) to the E330Q mutant.
an Ala did not significantly alter Ca\(^{2+}\) binding affinity for the enzyme, but it reduced \(k_{cat}\) by 61-fold and increased Man\(_9\)GlcNAc\(_2\) binding affinity by 50-fold. These data suggest that alterations in the \(-1\) subsite can lead to an increased binding affinity for an uncleaved substrate. The data also suggest that glycone interactions with the enzyme-bound Ca\(^{2+}\) ion, in the context of the appropriate tethering by Thr\(^{688}\), directly facilitate catalysis.

One of the main goals of our studies on ERManI was to map the energetics of interaction between glycan substrates and the active sites of class 1 mannosidases and to identify which residues contribute to catalysis and substrate specificity for members of the different enzyme subfamilies. The strategy for these studies will be to examine the individual contributions of enzyme residues in their interactions with the glycan substrates and the individual contributions of glycan residues in their interactions with the enzyme. The wild type enzyme is not the rapid on- and off-rates have contributions from both binding and ligand hydrolysis. A preferable model would be an effective model for SPR binding studies of this type, because the binding energies calculated from different combinations of glycans yielded reasonably similar energetic contributions (Table 5 and Fig. 3).

The results of the glycan binding studies revealed that the +1 subsite residue (residue M7) contributes a majority (~52\%) of the binding energy for a Man\(_9\)GlcNAc\(_2\) ligand, whereas the glycone binding to the \(-1\) subsite contributes only ~21% of the binding energy. Another ~19% of the binding energy is contributed from the other peripheral α1,2Man residues, and ~7.6% of the binding energy comes from core glycan residues. These data are consistent with binding data obtained from the respective ERManI mutants (Fig. 4). Mutations in the \(-1\) subsite or conditions that significantly reduce dMNJ binding affinity or catalysis either have a minimal effect on Man\(_9\)GlcNAc\(_2\) glycan binding (F659A mutant or Ca\(^{2+}\) depletion) or actually increase glycan binding affinity (T688A and E330Q mutants). Thus, the contributions of the \(+1\) subsite residues to the overall binding affinity can compensate for compromised interactions with glycan substrates in the \(-1\) subsite (Fig. 4).

In contrast, alterations in the \(+1\) subsite, such as the D463N or R461L mutants (Fig. 4), abolished glycan binding (\(K_d\) values >1 mM). The former side chain anchors the interactions between the enzyme and the M7 residue in the \(+1\) subsite by hydrogen bonding with the sugar O-3’ and O-4’ hydroxyls (46). For the latter residue, Arg\(^{643}\) has been shown to form a matrix of interactions with mannose residues M7, M4, and M3 (43, 61), yet substitution with an Ala residue (R461A) resulted in a near
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wild type binding affinity for Man₉GlcNAc₂. In contrast, the R461L mutant has no detectable Man₉GlcNAc₂ binding, confirming that there is likely a problem with steric hindrance in the latter mutant.

In conclusion, the combined studies on catalysis and glycan binding by wild type and mutant ERManI forms revealed an active site cleft that promotes substrate binding predominantly through interactions with the ≥ + 1 subsite residues (Fig. 4). How do these data compare with our emerging model for catalysis by class 1 α-mannosidases (46)? The docking of the glycone residue in a high free energy S⁻, conformation, predisposed for glycoside bond hydrolysis, is partly facilitated by van der Waals interactions with Phe₄₆₉ and additional hydrogen bonding to − 1 subsite residues. However, the predominant source for substrate binding energy is provided by interactions with the +1 residue, dominated by the pair of hydrogen bonds from Asp₁₆₉ to the 3⁻ and 4⁻- hydroxyls of the +1 subsite residue (Fig. 4). These latter interactions likely offset the entropic penalty for binding to a high free energy glycone conformation in the − 1 subsite, creating favorable energetics for the conformational distortion required for glycoside bond hydrolysis. Additional binding energy and branch specificity are provided by the interactions with the + > 1 subsite residues. Interactions between the glycone residue and the enzyme-bound Ca²⁺ influence the association and dissociation rates for glycan substrates, but do not significantly increase their respective equilibrium binding affinities. However, the Ca²⁺ ion does promote catalysis, presumably through the assistance of Thr₆₈₈, the sole residue involved in direct coordination with the divalent cation. In the absence of the Thr₆₈₈ side chain, a solvent water molecule likely replaces the lost point of Ca²⁺ ion coordination. Ca²⁺ binding affinity is not reduced in the T688A mutant, yet the catalytic rate is significantly reduced, and glycan binding affinity is surprisingly increased. These data strongly suggest that Thr₆₈₈ aids in Ca²⁺-mediated catalysis, either through appropriate positioning of the Ca²⁺ ion in the active site or by providing appropriate electrostatics to the divalent cation. It is worth noting that the water nucleophile in the inverting catalytic mechanism is directly coordinated to the Ca²⁺ ion (46), and an isosteric amide substitution of the adjoining general base residue (E599Q) reduced catalysis by 13,000-fold (Fig. 4) (46). However, this mutation did not increase the binding affinity for the uncleaved substrate. Thus, altering the electrostatics of the general base function for activation of the water nucleophile does not account for the increased glycan binding affinity for the T688A mutant. An alternative role for the Thr₆₈₈ side chain could be a mechanical tethering of the Ca²⁺ ion in a favorable position adjacent to the − 1 subsite that is required for efficient catalysis. A similar effect of reduced catalysis and increased binding of an uncleaved substrate is found for the general acid mutant E330Q. Future studies on the structures of co-complexes between the T688A and E330Q mutants with uncleaved substrates or substrate analogs should provide insights into the roles of the glycone conformational changes and the enzyme-associated Ca²⁺ ion in glycan hydrolysis.

The use of the E330Q mutant in SPR binding studies was also shown to be an effective tool in assessing the binding contributions of respective residues within the Man₉GlcNAc₂ substrate. Applying a similar approach to map the contributions of oligosaccharide substrate residues for the other Class 1 mannosidases will reveal the molecular basis of substrate recognition and specificity for this diverse enzyme family. More importantly, the ability to measure detailed binding affinities and kinetics using the equivalent of the E330Q mutant as a model should provide critical information for the analysis of new selective inhibitors for class I mannosidases as potential targets for human protein misfolding disorders.

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