Two-step Mechanism That Determines the Donor Binding Specificity of Human UDP-N-acetylhexosaminyltransferase*

Mack Sohpany, Jian Dong, and Masahiko Negishi

From the Pharmacogenetic Section, Laboratory of Reproductive and Developmental Toxicology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Received for publication, November 29, 2004, and in revised form, April 13, 2005
Published, JBC Papers in Press, April 14, 2005, DOI 10.1074/jbc.M413379200

In its x-ray crystal structures, α-1,4-N-acetylhexosaminyltransferase (exostosin-like protein 2 (EXTL2)) forms no direct interaction with the N-acetyl group of the UDP-N-acetylhexosamine. Mutation of the residues that interact with the hydroxyl groups of the donor not only failed to abrogate donor binding but in fact increased binding affinity. Isothermal titration calorimetry is now used to examine the binding nature of various UDP-sugars in H₂O and D₂O solutions. UDP-N-acetylhexosamines bind to ETLX2 with a high affinity in both solutions, resulting in a relatively large increase of entropy, whereas the weak binding of UDP-galactose and -glucose, which occurred only in D₂O solution, only slightly increased entropy. Thus, specific donor binding appears to undergo two distinct steps, beginning with the N-acetyl group expelling water from the donor-enzyme complex into the bulk solvent followed by positioning of the donor into the binding site for the subsequent interactions with the enzyme.

Glycosyltransferases comprise one of the largest families of enzymes found in nature. Their function is to catalyze the transfer of sugars from an activated donor substrate to various acceptor molecules, producing extremely diverse structures that are critical to a wide range of functions in all living organisms. These functions include storage of energy in the form of glycogen, synthesis of cell surface polysaccharides that mediate cell growth, cell-cell interactions, and the regulation of transcription of several genes (1–4). The x-ray crystal structures obtained to date have revealed that glycosyltransferases are similar in structure, with the N-terminal donor binding subdomain nearly identical in all of the structures (5–7). However, the underlying principles of glycosyltransferase substrate specificity remain unknown.

Human α-1,4-N-acetylhexosaminyltransferase (EXTL2), a member of the exostosin (EXT)-related family of enzymes, was originally characterized as the enzyme that transfers N-acetylglucosamine from UDP-GlcNAc to the nonreducing end of the common linker region of glucosaminylglycans, completing the specific linker region of the heparan chain (8). Recent x-ray crystal structures of ETLX2, solved in the presence of donor UDP-GlcNAc or UDP-GalNAc molecule, reveal that the sugar moiety of the donor forms only 4 hydrogen bonds with 4 enzyme residues, compared with the UDP moiety, which forms 15 hydrogen bonds with 10 enzyme residues (9). For those interactions with the sugar moiety, residue Arg-135 interacts with the 3-OH and 4-OH of GalNAc and GlcNAc molecules, respectively, whereas residue Arg-293 forms interactions with the 6-hydroxyl group of both molecules. In addition to its interaction with Arg-293, residue Asp-246 is within hydrogen bonding distance of the 3-hydroxyl group of the GlcNAc moiety. Most noticeably, however, the signature N-acetyl group of the donor sugar molecules resides in an open space and forms no interaction with the enzyme. Given the available structural information, ETLX2 provides an excellent model to investigate the molecular mechanism underlying specific donor-enzyme interactions. Here we used isothermal titration calorimetry (ITC) and enzyme kinetics to explore these interactions.

Glycosyltransferases...
RESULTS AND DISCUSSION

Isothermal titration calorimetry was utilized to study the nature of donor-EXTL2 interactions in solution. Fig. 1 shows the calorimetric profiles of two binding experiments, demonstrating the clear binding of UDP-GlcNAc but not UDP-Glc to wild type EXTL2. UDP, UDP-GlcNAc, and UDP-GalNAc were all found to bind to EXTL2 at a 1 to 1 ratio (Table I), which was consistent with the x-ray crystal structure showing only 1 donor molecule/EXTL2 molecule (9). The binding affinities were similar; the $K_d$ value of UDP binding was 23 μM, and those of UDP-GlcNAc and UDP-GalNAc were ~61 μM. ITC has been employed previously with α,1,3-galactosyltransferase, which displayed a $K_d$ of 60 μM for its binding to UDP-Gal (12). Contrary to our previous supposition that UDP-Gal and UDP-Glc would bind to EXTL2 (5), the present ITC data clearly demonstrate that neither bind (Table I). Thus, these observations provide direct evidence that despite having no direct interaction with the enzyme, the N-acetyl group determines the binding of UDP-N-acetylated hexosamines to EXTL2.

At the donor binding site, the N-acetylated hexosamine moiety adopts the conformation and orientation characteristic of substrates binding to glycosyltransferases, rotating at the C-1 atom to bend the unit under the UDP moiety and expose it to the hydroxyl group of the acceptor substrate (10). These characteristics are conserved in the binding of UDP-GlcA and UDP-Gal in the x-ray crystal structures of β-1,3-glucuronsyltransferase and α,1,4-galactosyltransferase, respectively (5, 12–14). The binding conformation and orientation are supported by the interactions of the hydroxyl groups of the N-acetylated hexosamine moiety with residues (e.g. Arg-135, Arg-293, and Asp-246) at the active site of EXTL2 (9). The two Arg residues are in position to form hydrogen bonds with the hydroxyl groups of the GlcNAc moiety, whereas the Asp residue is interacting with Arg-293. In mouse and human EXT1, EXT2, EXTL1, EXTL2, and EXTL3, Arg-135 and Asp-246 are invariant, with Arg-293 only differing in the mouse and human EXTL1 sequence. Arg-135 forms a characteristic “triad”-like interaction with the 3-OH group of GlcNAc and the side chain of Asp-151, the first Asp of the signature motif DxD in all glycosyltransferases (5). These characteristic triads are observed in the known structures of other glycosyltransferases (5). To examine whether these residues play a role in the binding of UDP-N-acetylated hexosamines, their alanine mutants, known to abrogate transfer activity (9), were subjected to ITC experiments.

The resulting supernatants were subjected to a Hi-Load 16/60 Superdex 30-pg column (Amersham Biosciences) equilibrated with 250 mM NH₄HCO₃ containing 7% 1-propanol using the AKTA purifier (Amersham Biosciences). Fractions were collected at a rate of 1 ml/min, and the amount of the hydrolyzed product was quantified with a scintillation counter. Data analysis was performed using the Origin version 7.0 software programmed to fit data to an (number of sites), $K_d$ value and $V_{max}$ value using Michaelis-Menten kinetics. $K_{app}$ values for UDP-GlcNAc hydrolysis by EXTL2 were determined at 20, 30, 40, and 50 °C by using the final concentrations of UDP-GlcNAc (3.2, 6.4, 9.6, 12.8, and 16 μM). Entropy was calculated by plotting log $K_{app}$ versus 1/degrees kelvin (11).

fig. 1. calorimetric profiles of the binding of UDP-GalNAc and UDP-Gal to wild type EXTL2 in H2O solution. A, the reaction cell contained a solution of EXTL2 (1.4 ml, 165.4 μM) in 25 mM HEPES buffer, pH 7.5, containing 20 mM MnCl₂, 0.1 M NaCl, and 1 mM CaCl₂. The syringe contained 4 mM UDP-GalNAc in the same buffer. Top, raw calorimetric data obtained from the injection of 6-μl aliquots of UDP-GalNAc at 3-min intervals into 1.4 ml of EXTL2. Bottom plot shows the integrated binding isotherm with the experimental points (■) and best fit. The bottom plot also includes the final fitting parameters for the best fit, providing values for $n$ (number of sites), $ΔH$ (cal/mol), $ΔS$ (cal/mol/degree), and $K$ (binding constant in M⁻¹). No fitting was possible in B; no curve was generated because of the lack of binding activity.
The results of the ITC experiments (Table II) clearly demonstrate that all of the mutants bind to UDP-N-acetylgalactosamines at pH 7.5 and that these hydrophobic binding interactions between the N-acetylgalactosamine and EXTL2 do not appear to be essential for binding. However, their $K_d$ values for donor binding are also lower than that of wild type EXTL2, suggesting that these residues can be determining factors for binding affinity. EXTL2 hydrolyzes UDP-GlcNAc at a $K_d$ value that is similar to its $K_v$ value (Table II). Consistent with their $K_d$ values, the mutants also decrease their $K_m$ values for the hydrolysis of UDP-GlcNAc, and, moreover, they exhibit similar $K_d$ and $K_m$ values. Because these residues that interact with the $N$-acetylgalactosamine moiety are all charged residues, ITC experiments were performed at pH 5.0 and 9.0 to confirm the role that charge plays in determining the affinity of donor binding. The affinity of wild type EXTL2 increases 10-fold at pH 5.0 compared with the original value at pH 7.5. Conversely, the mutants only slightly alter their binding affinity to UDP-GlcNAc at pH 5.0; among the mutants, the R135A mutant decreases the binding affinity 1.7-fold, whereas the D246A mutant decreases the binding affinity 2.5-fold, whereas the D246A mutant displays the highest increase of 2.5-fold, whereas the D246A mutant decreases the binding affinity 1.7-fold.

Enzyme function can be driven by changes in enthalpy or entropy. When driven by a change in enthalpy, catalysis is initiated by reducing the heat of activation (15). Conversely, in the mechanism driven by a change in entropy, enzymes properly position substrates and/or exclude water within their active sites allowing for the reaction to proceed; a process first described by Koshland (16) and later dubbed the “entropy trap” by Westheimer (17). This mechanism works by overcoming the unfavorable entropy of activation usually inherent in a chemical reaction (15–17). As indicated by the fact that the absolute $\Delta S$ values multiplied by the temperature of the reaction ($T \Delta S$) are larger than the absolute $\Delta H$ values (Table I), the binding reactions of UDP-GlcNAc and UDP-GalNAc are driven by a change in entropy. The N-acetyl group is the determining factor for this increase of entropy in the binding of UDP-N-acetylgalactosamines to EXTL2. This is further supported by the fact that UDP binding only slightly (one-tenth of the UDP-N-acetylgalactosamines) increases entropy and is driven by a change in enthalpy. Because increases in entropy in systems at constant temperature are strongly correlated with the loss of previously oriented waters of hydration (18), the N-acetyl group may expel water from the area of enzyme-donor complex into the bulk solvent to position UDP-N-acetylgalactosamine within the binding site.

![Fig. 2. pH-dependent alteration of $V_{max}$ values of wild type and mutant EXTL2. Fifty-three-μg of protein were tested in an assay mixture containing 25 mM HEPES, pH 7.5, 20 mM MnCl$_2$, 0.1% (v/v) Triton X-100, and 1 μM of UDP-[3H]GlcNAc (36.0 Ci/mmol) (PerkinElmer Life Sciences). The pH of the HEPES buffer was then adjusted accordingly. Assay mixtures containing radioactive plus nonradioactive UDP-GlcNAc at final concentrations of 16.0, 28.0, 40.0, 52.0, and 80.0 μM were incubated for 1 h at 37 °C. Hydrolyzed product was collected and quantified with a scintillation counter. $V_{max}$ values were then calculated using Michaelis-Menten kinetics and then plotted against the pH values of their respective reaction buffers.](image-url)
This analysis produced a similar X-100, and 1 °C was performed to obtain an obtained from the ITC experiments, kinetic analysis of the hydrolyzed product was collected and quantified with a scintillation counter. Velocity was calculated by plotting log $K_{eq}$ versus 1/degrees kelvin.

Thermodynamic parameters, $\Delta G$ (cal/mol), $\Delta H$ (cal/mol), $\Delta S$ (cal/mole/degree), and $n$ (number of binding sites), for donor binding with wild type EXTL2 in D$_2$O solution at 303 K

Substrate and EXTL2 concentrations were 4 mM and 85 μM, respectively.

| Substrate   | $\Delta G$ | $\Delta H$ | $\Delta S$ | $K_d$  | n   |
|-------------|------------|------------|------------|-------|-----|
| UDP-GalNac  | $-6083.4 \pm 221.1$ | $-3014 \pm 221.1$ | 9.496 | 44.19 | 1.279 |
| UDP-GlcNac  | $-6038.3 \pm 474.2$ | $-3161 \pm 474.2$ | 9.773 | 66.14 | 1.094 |
| UDP-Gal     | $-5214.2 \pm 66.14$ | $-4374 \pm 66.14$ | 9.849 | 44.19 | 1.279 |
| UDP-Glc     | $-5097.1 \pm 61.01$ | $-4958 \pm 61.01$ | 9.684 | 66.14 | 1.094 |

Donor Recognition of UDP-N-acetylhexosaminyltransferase

Donor Recognition of UDP-N-acetylhexosaminyltransferase

Table III

Thermodynamic parameters, $\Delta G$ (cal/mol), $\Delta H$ (cal/mol), $\Delta S$ (cal/mole/degree), and $n$ (number of binding sites), for donor binding with wild type EXTL2 in D$_2$O solution at 303 K

Substrate and Y193A concentrations were 2 mM and 41 μM, respectively. ND, no detectable binding.

| Substrate   | $\Delta G$ | $\Delta H$ | $\Delta S$ | $K_d$  | n   |
|-------------|------------|------------|------------|-------|-----|
| UDP-GalNac  | $-5917.7 \pm 123.1$ | $-981.8 \pm 123.1$ | 16.29 | 53.79 | 0.909 |
| UDP-GlcNac  | $-6136.9 \pm 70.93$ | $-788.9 \pm 70.93$ | 17.65 | 37.44 | 0.812 |
| UDP-Gal     | ND         | ND         | ND         | ND    | ND  |
| UDP-Glc     | ND         | ND         | ND         | ND    | ND  |

Table IV

Thermodynamic parameters, $\Delta G$ (cal/mol), $\Delta H$ (cal/mol), $\Delta S$ (cal/mole/degree), and $n$ (number of binding sites), for UDP-GlcA binding with wild type EXTL2 and mutants at 303 K

Substrate and EXTL2 concentrations were 4 mM and 40 μM, respectively. ND, no detectable binding.

| Enzyme  | $\Delta G$ | $\Delta H$ | $\Delta S$ | $K_d$  | n   |
|---------|------------|------------|------------|-------|-----|
| WT$^1$  | $-6283.3 \pm 161.3$ | $-1805 \pm 161.3$ | 14.78 | 29.41 | 0.905 |
| WT$^2$  | $-7131.1 \pm 39.26$ | $-1971 \pm 39.26$ | 17.03 | 7.17  | 1.106 |
| R293A   | $-6323.7 \pm 34.85$ | $-2209 \pm 34.85$ | 13.58 | 27.43 | 0.780 |
| D246A   | ND         | ND         | ND         | ND    | ND  |
| R135A   | ND         | ND         | ND         | ND    | ND  |

Table V

Thermodynamic parameters, $\Delta G$ (cal/mol), $\Delta H$ (cal/mol), $\Delta S$ (cal/mole/degree), and $n$ (number of binding sites), for UDP-GlcA binding with wild type EXTL2 and mutants at 303 K

Substrate and Y193A concentrations were 2 mM and 41 μM, respectively. ND, no detectable binding.

| Enzyme  | $\Delta G$ | $\Delta H$ | $\Delta S$ | $K_d$  | n   |
|---------|------------|------------|------------|-------|-----|
| WT$^1$  | ND         | ND         | ND         | ND    | ND  |
| WT$^2$  | ND         | ND         | ND         | ND    | ND  |

 ompounds are critically involved in the binding of EXTL2 to UDP-N-acetylhexosamines. Further evidence substantiating the role of water as well as that of the N-acetyl group in binding was obtained from the findings that both UDP-Gal and UDP-Glc were capable of binding to EXTL2 in D$_2$O solution (Table III). Although their affinities ($K_d$ values) to EXTL2 were still 4–5-fold weaker than those of the UDP-N-acetylhexosamines, the binding of UDP-Gal and UDP-Glc was accompanied with a very slight increase (nearly zero $\Delta S$) in entropy. No hydrolysis of donor substrate occurs in D$_2$O solution; thus the $\Delta S$ values obtained from the ITC experiments in D$_2$O solution are solely the result of donor binding. To corroborate the $\Delta S$ values obtained from the ITC experiments, kinetic analysis of the hydrolysis reaction by EXTL2 at 20 and 50 °C was performed to obtain an $\Delta S$ value for the hydrolysis. This analysis produced a similar $\Delta S$ value (17.83 μM) to those produced by ITC experiments (Fig. 3). This similar and positive $\Delta S$ value for the hydrolysis further substantiates the characteristic nature of donor binding. Moreover, it is reasonable to conclude that the binding and proper positioning of the donor substrate may be all that is necessary for a reaction to occur. In principle, water can be involved in intermolecular interactions through two processes: by being expelled into the bulk solvent from the site of interaction or by assuming an ordered position within the active site to mediate the interaction (18). Quiocho et al. (20) have already shown that a bound water molecule determines the sugar specificity of l-arabinose-binding protein. In fact, re-examination of the x-ray crystal structures of EXTL2 reveals a water molecule that is in position to form hydrogen bonds with the phenolic OH of Tyr-193 and the N-acetyl group of UDP-GlcNac (but not UDP-GalNac) (9). We mutated this residue to alanine and found that the Y193A mutant fully retains binding ability (with nearly identical values for $K_d$ and $\Delta S$) to UDP-N-acetylhexosamines (Table IV). Thus, this water and its bridging interaction with the N-acetyl group and the phenolic OH are not required for the binding of UDP-N-acetylhexosamines to EXTL2 nor are they the reason for UDP-Gal and UDP-Glc not being able to bind to this enzyme.

GlcA is another modified monosaccharide component of the heparan chain, and it has been suggested that UDP-GlcA is incapable of binding to EXTL2 because the 6-carboxyl group of the GlcA moiety would clash with the conserved residues within the binding pocket of EXTL2 (5). Contrary to this suggestion, the present results from ITC experiments clearly show that UDP-GlcA binds to EXTL2 with almost identical entropy and a stronger affinity to those of the UDP-N-acetylhexosamines in H$_2$O solution (Table V). With respect to its binding activity, UDP-GlcA appears to expel water like the UDP-N-acetylhexosamines as suggested by its positive $\Delta S$ value. Additionally, UDP-GlcA also binds to EXTL2 in D$_2$O solution; its binding in D$_2$O is also accompanied with an increase in en-
tropy, suggesting that the carboxyl group may be facilitating this binding activity in the same manner as the N-acetyl group does for the UDP-N-acetylhexosamines. Contrary to the binding of UDP-N-acetylhexosamines, that of UDP-GlcA significantly increased the binding affinity ($K_d$), whereas the entropy of binding remained identical to that in H$_2$O solution (Table V). Despite its binding capability, UDP-GlcA is not hydrolyzed by EXTL2. Consistent with this capability, however, UDP-GlcA does inhibit the hydrolysis of UDP-N-acetylhexosamines by EXTL2 (data not shown). In addition to wild type EXTL2, UDP-GlcA binds to the R293A mutant, whereas it cannot bind to the mutants R135A and D246A. The binding to the R293A mutant but not to the R135A and D246A mutants indicates that the GlcA molecule may adopt the same conformation and orientation of the N-acetylhexosamines at the binding site, resulting in directing the 6-carboxyl group toward Arg-135 and Asp-246. Although the x-ray crystal structure of an EXTL2-UDP-GlcA complex should provide us with more insight of the molecular basis for UDP-GlcA binding in the future, these results support the hypothesis that the increase in entropy caused by UDP-GlcA binding is the general mechanism that enables modified monosaccharides to bind to glycosyltransferases.

The data presented in this article suggest that the expulsion of water from the donor-EXTL2 complex into the bulk solvent positions the donor to form the hydrogen bond with the conserved residues of EXTL2. Thus, the water expulsion by the N-acetyl group determines the donor specificity of EXTL2. Subsequent interactions allow for the donor molecule to adopt the proper conformation, orientation, and affinity within the active site so as to confer the enzyme with catalytic capability. Although the mechanism by which modifying groups such as N-acetyl and 6-carboxyl groups expel water is unknown at the present time, the characterization of water expulsion as the determining factor for donor substrate specificity suggests that there may be a water barrier that prevents the ligand from moving into the binding site of the protein, and the expulsion of water by modifying groups as the mechanism for donor recognition has uncovered a new concept in understanding molecule-molecule interactions. Our interpretation of the ITC data is also based upon the x-ray crystal structures of EXTL2 in which no specific interaction between the NAc groups with EXTL2 is present. If such an interaction occurs in solution, the underlying mechanism of donor recognition and catalysis may not be understood only by the observed increase in $\Delta S$ associated with the modifying groups. The concept of entropy-driven mechanisms for enzyme functions is not a novel one, nor is it limited to EXTL2. Recently, ribosome-catalyzed peptide bond formation and the function of cytidine deaminase have been reported to utilize the entropy trap to process their reactions (15, 21). Clearly, the role of entropy and substrate positioning in enzymes and the advantages and disadvantages of using such a mechanism is an area ripe for further exploration.

REFERENCES

1. Perrimon, N., and Bernfield, M. (2000) Nature, 404, 725–728
2. Forssberg, E., and Kjellen, L. (2001) J. Clin. Invest. 108, 175–180
3. Eko, J. D., and Sellick, S. B. (2002) Annu. Rev. Biochem. 71, 455–471
4. Hu, Y., and Walker, S. (2002) Chem. Biol. 9, 1287–1296
5. Negishi, M., Dong, J., Darden, T. A., Pedersen, L. G., and Pedersen, L. C. (2003), Biochem. Biophys. Res. Commun. 303, 393–398
6. Tarbouriech, N., Charnock, S. J., and Davies, G. J. (2001) J. Mol. Biol. 314, 655–661
7. Ungiligi, U. M., and Rini, J. M. (2000) Curr. Opin. Struct. Biol. 10, 510–517
8. Kitagawa, H., Shimakawa, H., and Sugahara, K. (1999) J. Biol. Chem. 274, 13933–13937
9. Pedersen, L. C., Dong, J., Taniguchi, F., Kitagawa, H., Krabn, J. M., Pedersen, L. G., Sugahara, K., and Negishi, M. (2003) J. Biol. Chem. 278, 14420–14428
10. Presse, W. H., Flannery B. P., Teukolsky, S. A., and Vetterling, W. T. (1989) Numerical Recipes in FORTRAN: The Art of Scientific Computing, Cambridge University Press, Cambridge, UK
11. Segel, Irwin H., (1976) Biochemical Calculations, 2nd Ed., John Wiley & Sons, Inc., New York
12. Boix, E., Zhang, Y., Swaminathan, J., Brew, K., and Achaya, K. R. (2002) J. Biol. Chem. 277, 28310–28313
13. Pedersen, L. C., Darden, T. A., and Negishi, M. (2002) J. Biol. Chem. 277, 21869–21873
14. Persson, K., Ly, H. D., Dieckelmann, M., Wakarechuk, W. W., Withers, S. G., and Strynadka, N. C. J. (2001) Nat. Struct. Biol. 8, 166–174
15. Sievers, A., Beringer, M., Rodina M. V., and Davies, G. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 101, 7897–7901
16. Koehl, D. R. (1980) Adv. Enzymol. 22, 45–97
17. Westheimer, F. H. (1962) Adv. Enzymol. 24, 441–482
18. Hu, I. C., Darden, T. A., and Negishi, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 101, 7897–7901
19. Koshland, D. E. (1960) Adv. Enzymol. 22, 45–97
20. Kushner, D. J., Baker, A., and Dunstall, T. G. (1999) Can. J. Physiol. Pharmacol. 77, 79–88
21. Snider, M. J., Lazarevic, D., and Wolfenden, R. (2002) Biochemistry 41, 3925–3930