Circular Dichroic Spectroscopy of N-Acetylgalcosaminyltransferase V and Its Substrate Interactions*

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β-1,6-N-Acetylgalcosaminyltransferase V (EC 2.4.1.155) catalyzes the transfer of N-acetylgalcosamine (GlcNAc) from UDP-GlcNAc in β(1,6)-linkage to the α(1,6)-linked mannose of N-linked oligosaccharides. Circular dichroism (CD) was used to investigate the secondary structure of a recombinant, soluble form of the enzyme and its interaction with UDP-GlcNAc and an inhibitory substrate analog. The CD spectrum of the apoenzyme indicated the presence of small amounts of β-structure and substantial amounts (>50%) of α-helicity. The CD spectra of solutions containing UDP-GlcNAc and different ratios of UDP-GlcNAc-enzyme were measured. Interestingly, the spectrum of each mixture could not be accounted for by simple additivity of the two individual spectra, indicating a change in environment of the chromophores and/or a conformational change of the substrate or protein concomitant with binding. Similar results were obtained with mixtures of UDP and the enzyme. Analysis of the CD difference spectra at three wavelengths yielded an estimated average Kd of 4.4 mM for UDP-GlcNAc and 3.8 mM for UDP. By contrast, addition of the CD spectrum of an inhibitory substrate analog of its oligosaccharide acceptor substrate and the CD spectrum of the enzyme could account for that observed of an inhibitor-enzyme mixture; moreover, addition of the inhibitor to a mixture of UDP-GlcNAc and enzyme did not alter the Kd associated with UDP-GlcNAc binding to the enzyme. These results and kinetic studies reported herein suggest an ordered reaction in which UDP-GlcNAc binds first to the enzyme, followed by the sequential binding of the trisaccharide substrate.

Glycosyltransferases transfer sugars from sugar-donor substrates to acceptor substrates and synthesize complex carbohydrates (1). In animal cells, these enzymes are normally membrane-bound and present in relatively low copy number per cell, although many of the enzymes have been detected in soluble form in serum and cerebrospinal fluid after release from Golgi membranes by proteolysis (2). The development of affinity chromatography techniques (3–5) has allowed many glycosyltransferases to be purified and the cDNAs, which encode them, to be isolated and characterized, although several glycosyltransferase cDNAs have been isolated by expression cloning techniques (6–8). Although the amino acid sequences of many of these enzymes are now known, none of the enzymes has as yet been studied by x-ray crystallography techniques; consequently, very little is known of their secondary and tertiary structure. Several studies, however, have used modified substrates or differential labeling techniques to localize regions in the primary sequence of particular glycosyltransferases, which are in proximity to their active sites or are involved in catalysis (9, 10). Although short sequence motifs are shared between a few enzymes in this family (cf. Refs. 11 and 12), little information is available concerning the physical interaction between any of these enzymes and its donor and acceptor substrates. A logical tool to utilize in the study of glycosyltransferase-substrate interactions is that of circular dichroism.

We have recently isolated a cDNA encoding N-acetylgalcosaminyltransferase V (GlcNAc-TV, EC 2.4.1.155) and engineered it such that when transfected into Chinese hamster ovary cells, a soluble form of the enzyme is secreted into the culture medium (11, 12). Quantities sufficient for CD studies of this form of the enzyme can, therefore, be produced and purified to homogeneity. This glycosyltransferase transfers N-acetylgalcosamine (GlcNAc) from UDP-GlcNAc to an oligosaccharide acceptor substrate by the following reaction:

\[
\begin{align*}
\text{GlcNAc} + \text{GlcNAc} &\rightarrow \beta(1,2)\text{Man}\alpha(1,6)\text{Man-R} \\
\text{UDP-GlcNAc} \quad \xrightarrow{\beta(1,6)} \quad \text{GlcNAc-T V} \\
\text{UDP} \\
\end{align*}
\]

GlcNAc-T V is of particular interest because the regulation of its expression appears to be controlled by several mechanisms (12–14). In particular, its expression is significantly stimulated when cells are transformed by oncogenes, and the expression of its cell surface oligosaccharide products on specific glycoproteins regulates, among other structures, the expression of polylactosamine on N-linked oligosaccharides (15–17). Polylactosamine is bound with high affinity by the family of animal lectins known as the galectins (18) and has been shown to function in cell adhesion (19).

In the present study, the CD spectra of recombinant, soluble GlcNAc-T V was studied in the absence and presence of its donor substrate, UDP-GlcNAc, an inhibitory substrate analog of its oligosaccharide acceptor substrate (20), and the competitive inhibitor UDP. The CD spectra of both enzyme-UDP-GlcNAc and enzyme-UDP mixtures could not be accounted for by additivity of the individual spectra, thus showing that CD is useful for studying GlcNAc-T V-UDP-GlcNAc complexes. Since, in the absence of oligosaccharide acceptor, UDP-GlcNAc and UDP bind to enzyme in a reversible bimolecular interac-

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‡ The abbreviations used are: GlcNAc-TV, N-acetylgalcosaminyltransferase V; GlcNAc-T I, N-acetylgalcosaminyltransferase I; GlcNAc-T II, N-acetylgalcosaminyltransferase II.
tion, the CD data were analyzed with the binding model, \( E + S \rightleftharpoons ES \), and yielded calculated average \( K_d \) values of 4.4 and 3.8 mM, respectively. Interestingly, the CD spectrum of a mixture of the enzyme and a trisaccharide acceptor substrate analog, which functions as a competitive inhibitor, could be fully accounted for by the simple sum of the individual spectra; moreover, the presence of the inhibitor had no effect on the \( K_d \) of UDP-GlcNAc binding to GlcNAc-T V.

EXPERIMENTAL PROCEDURES

Materials and Recombinant Enzyme—UDP-GlcNAc and UDP were products of Sigma, and the inhibitory trisaccharide acceptor analog, \( b \)-O-(2-acetamido-2-deoxy-\( \beta \)-D-glucopyranosyl)-6-deoxy-\( b \)-manopyranosyl-\( \beta \)-D-glucopyranoside, was obtained from Dr. Ole Hindsgaul (University of Alberta, Canada (21)). Recombinant, soluble GlcNAc-T V was purified from concentrated Chinese hamster ovary cell medium (12). After extensive dialysis against the buffer used for CD spectroscopy, 10 mM Tris-HCl, pH 6.5, containing 0.1 mM EDTA, the enzyme was concentrated at 3000 \( g \) using Centricon-30 columns (Amicon).

CD Spectra of GlcNAc-T V in the Presence and Absence of UDP-GlcNAc, UDP, and Trisaccharide Inhibitor—Solutions of either 3.2 or 3.6 \( \mu \)M GlcNAc-T V were scanned in a Jasco J-700 spectropolarimeter at ambient temperature using a cell of 1 mm pathlength. Replicate scans were obtained at 0.2 nm resolution, 0.2 nm bandwidth, and a scan speed of 50 nm/min, followed by spectral averaging and smoothing. Conditions were such that the signal at 220 nm was about 20 millidegrees with enzyme alone; the variation from replicate scans was at most \( \pm 1 \) millidegree. CD spectra of GlcNAc-T V plus UDP-GlcNAc or plus UDP were determined by titrating a concentrated solution of the sugar nucleotide or nucleotide into the enzyme solution, and control spectra were obtained on UDP-GlcNAc or UDP solutions at the same concentrations. The CD spectrum of GlcNAc-T V was also measured in the presence of the trisaccharide inhibitor, which too was scanned in the absence of enzyme. Lastly, CD spectra were determined on mixtures of GlcNAc-T V (3.6 \( \mu \)M), trisaccharide inhibitor (1 mM), and various concentrations of UDP-GlcNAc (1–10 mM). The signal-to-noise ratio of all spectra was satisfactory above 205 nm; data were not analyzed below 200 nm due to low signal-to-noise ratios.

Enzymatic Activity Assay—The radiochemical assays were performed as described using the standard synthetic trisaccharide acceptor (20). The acceptor concentration was varied from 5 \( \mu \)M to 0.2 mM, and the UDP-GlcNAc donor concentration varied from 0.1 to 4 mM.

RESULTS

The CD spectrum of GlcNAc-T V between 200 and 260 nm is shown in Fig. 1. The negative extrema located at 208 and 222 nm are indicative of \( \alpha \)-helicity. When analyzed by the Convex

Constraint Analysis program (22), the spectrum is consistent with the presence of 58% helicity, 5% \( \beta \)-structure, and 37% aperiodic conformation. The secondary structure estimates from CD analysis were compared with those predicted by a neural net system using the amino acid sequence of the truncated enzyme (23). The latter predicted 45% helicity, 11% \( \beta \)-structure, and 44% aperiodic conformation, in reasonable agreement with the experimental findings.

Since UDP-GlcNAc is but one of two required substrates of the enzyme, it is possible to form stable enzyme-substrate complexes without catalysis occurring. First, various concentrations of UDP-GlcNAc (1–200 \( \mu \)M) were scanned in the 1-mm cell, but no detectable ellipticity was observed between 200 and 300 nm (data not shown). At higher concentrations, however, negative ellipticity was measured between 200 and 250 nm, while positive ellipticity was noted between 250 and 300 nm (Fig. 2). A concentration-dependent change in the CD spectrum was noted above 0.5 mM. For example, at 1 mM UDP-GlcNAc the CD spectrum is characterized by negative and positive extrema associated with broad bands at about 214 and 270 nm, respectively; at 10 mM distinct and well resolved negative and positive bands appear at about 231 and 285 nm, respectively.
In addition, at the higher concentrations of UDP-GlcNAc there are negative CD bands between 200 and 225 nm and only minimal positive ellipticity between 250 and 280 nm. Similar spectral results were observed with UDP and may result from dimerization or stacking of the nucleotides at these higher concentrations.

The CD spectrum of a mixture of GlcNAc-TV (3.6 \( \mu M \)) and UDP-GlcNAc was found to be quite different from that of the mixture with enzyme and inhibitor at the same concentrations. To facilitate data manipulation and comparison, the spectra are given simply as the difference ellipticity, \( \delta \theta \). UDP-GlcNAc concentration: \( a \), 0.2 mM; \( b \), 0.5 mM; \( c \), 1 mM; \( d \), 2 mM; \( e \), 3 mM; \( f \), 4 mM.

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The CD spectrum of a mixture of GlcNAc-TV (3.6 \( \mu M \)) and UDP-GlcNAc (4 mM) is shown in Fig. 3, along with reference spectra of enzyme and substrate alone. The sum of the spectra of GlcNAc-TV and UDP-GlcNAc was found to be quite different from that of the mixture (Fig. 3), establishing that this system does not obey simple additivity rules. Comparable studies were then performed at various concentrations of UDP-GlcNAc and a fixed enzyme concentration. The CD spectrum difference, i.e., the spectrum of the mixture minus the sum of the spectra of enzyme and substrate, changed with increasing concentrations of UDP-GlcNAc between 0.2 and 2 mM; at 3 and 4 mM UDP-GlcNAc, the CD spectral differences were essentially identical (Fig. 4). At the lower concentrations of substrate (0.2–2 mM), the CD difference extremum was negative and centered at about 210 nm; at about 250 nm the difference ellipticity was slightly positive. The apparent limiting CD difference spectrum, e.g., occurring at 3, 4, and 10 mM UDP-GlcNAc, is characterized by negative extrema at about 210 and 216–218 nm, with a weak positive extrema at about 276 nm. Measurements were then made to determine if the non-additivity noted above could be observed using UDP alone, since this nucleotide is a competitive inhibitor of the enzyme (24). The results (Fig. 5) demonstrated that mixtures of UDP and enzyme also yield spectra that fail to follow simple spectral additivity, similar to the findings obtained with mixtures of UDP-GlcNAc and enzyme.

An inhibitory substrate analog of the enzyme has been synthesized in which the 6'-OH of the \( \alpha(1,2) \)-linked mannose of the trisaccharide acceptor, to which GlcNAc is transferred by the enzyme, has been replaced by hydrogen (20). This analog displays a \( K_i \) of 60 \( \mu M \), compared to the \( K_m \) of 213 \( \mu M \) for the

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**FIG. 4.** CD difference spectra of GlcNAc-TV (3.6 \( \mu M \)) and various concentrations of UDP-GlcNAc. These spectra were generated by subtracting the sum of the individual spectra, i.e., enzyme plus inhibitor, from that of the mixture with enzyme and inhibitor at the same concentrations. To facilitate data manipulation and comparison, the spectra are given simply as the difference ellipticity, \( \delta \theta \). UDP-GlcNAc concentration: \( a \), 0.2 mM; \( b \), 0.5 mM; \( c \), 1 mM; \( d \), 2 mM; \( e \), 3 mM; \( f \), 4 mM.

**FIG. 5.** CD spectra of GlcNAc-TV (3.2 \( \mu M \)); UDP (3.75 mM); and a mixture of the two (c) at the same concentrations. Also shown is an additive spectrum (d) of enzyme plus inhibitor, i.e., the addition of the two individual components. The results are given as ellipticity to facilitate comparisons.

**FIG. 6.** CD spectra of enzyme (a), 3.6 \( \mu M \); the trisaccharide inhibitor (b), 1 mM; and a mixture of the two (c) at the same concentrations. Also shown is the additive spectrum of enzyme plus inhibitor (d), which overlaps with that of the experimentally determined spectrum. As before, the results are given as ellipticity to facilitate comparisons.

**FIG. 7.** CD spectra of a mixture of enzyme (3.6 \( \mu M \)) plus UDP-GlcNAc (a), 1 mM; trisaccharide inhibitor (b), 1 mM; and a mixture of enzyme plus UDP-GlcNAc plus trisaccharide inhibitor (d), which is quite similar to that of the spectrum of the three-component mixture. Measured ellipticities are given to aid in comparison of the various spectra.
trisaccharide acceptor, determined using crude enzyme from baby hamster kidney cells. This inhibitory acceptor analog was chosen because it could be mixed simultaneously with the enzyme and UDP-GlcNAc without a catalytic reaction. Interestingly, mixtures of the enzyme with the trisaccharide inhibitor, characterized by a negative extremum at 210 nm, could be fully accounted for by the individual spectra (Fig. 6). The CD spectrum of the mixture of enzyme (3.6 μM), UDP-GlcNAc (1 mM), and inhibitor (1 mM) is, within experimental error, identical to the additive spectra of enzyme plus UDP-GlcNAc and inhibitor (Fig. 7), indicating that the inhibitor does not alter the interaction of GlcNAc-TV and UDP-GlcNAc.

Since CD spectroscopy can be used to follow enzyme-UDP-GlcNAc interactions, the CD difference spectra were analyzed to obtain information on the Kd of the reaction, E + S ⇔ ES. We have defined the ellipticity at any wavelength of enzyme, substrate, and enzyme-substrate mixture as θGlcNAc-TV, θUDP-GlcNAc, and θmixture, respectively, and the difference ellipticity as δθ, where δθ = θmixture - θGlcNAc-TV - θUDP-GlcNAc. For a reversible bimolecular reaction, E + S ⇔ ES, the Kd is given by [E] [S]/[ES], where brackets denote concentrations of the indicated species. Since [E], [S] = [E] + [S] and [S] = [S] + [ES], where [E], and [S], refer to total enzyme and substrate concentrations, respectively, the Kd can be written as follows:

$$K_d = ([E] - [ES])[S]_t - [ES]/[ES] \quad (Eq. 1)$$

If we assume that δθ is directly proportional to [ES] and denote δθmax as the limiting δθ that occurs when the enzyme is saturated in a presumed 1:1 stoichiometry with substrate, then, under our experimental conditions where [S]t >> [E], and consequently [S]t >> [ES] at substrate concentrations corresponding to the spectral differences, Equation 1 yields the following approximate relationship:

$$δθ = (δθ_{max}[S]/[K_d + [S]]) \quad (Eq. 2)$$

We fit δθ versus [S]t at three wavelengths, 208, 215, and 222 nm, and solved each set of data for Kd and δθmax using Equation 2 and a Prism program (Fig. 8). The results (mean ± S.E.) for the Kd values at the three wavelengths were, respectively, 4.9 ± 2.7, 3.5 ± 1.1, and 4.9 ± 3.3 mM. Since there is no statistical difference between these values, we can calculate an average Kd of 4.4 mM for the binding of UDP-GlcNAc to GlcNAc-TV. Similar calculations using Equation 2 for the interaction of enzyme and UDP yielded a Kd value of 3.8 mM (data not shown).

To characterize further the kinetic reaction mechanism of GlcNAc-TV, activity was measured at various concentrations of UDP-GlcNAc and trisaccharide acceptor. The enzymatic activity assay data were then plotted as the reciprocal of the activities versus the reciprocal of the substrate concentrations (25). In Fig. 9A, for each concentration of UDP-GlcNAc, the reciprocal of activities at different concentrations of trisaccharide acceptor were plotted against the reciprocals of substrate concentrations.
The CD spectra of mixtures of GlcNAc-T V and various concentrations of its substrate, UDP-GlcNAc, could not be accounted for by simple additivity of the spectrum of each component alone. Since both the enzyme and the substrate are optically active and exhibit overlapping bands between 200 and 250 nm, it is difficult to attribute with confidence the spectral differences to just one of the species. The problem is exacerbated in the case of UDP-GlcNAc, which shows a dramatic concentration-dependent change in its CD spectrum, even in the absence of enzyme. Nevertheless, despite these caveats, it was possible to analyze the spectral data and obtain an average \(K_p\) of 4.4 mM for the equilibrium binding of UDP-GlcNAc to GlcNAc-T V. The nucleotide moiety, UDP, also showed this anomalous concentration-dependent change in its CD spectrum and caused non-additive spectral changes, similar to those observed with UDP-GlcNAc and enzyme. Calculation of an average \(K_p\) for UDP (3.8 mM) yielded a value similar to that for UDP-GlcNAc binding to the enzyme. The CD spectra of UDP and enzyme mixtures appear to be very similar to those observed with mixtures of UDP-GlcNAc and enzyme, suggesting that the active site binding environments of UDP and UDP-GlcNAc are similar. A comparison of the \(K_p\) of UDP-GlcNAc, 6 mM, and the \(K_p\) for UDP, 2 mM, however, reveals a difference in these kinetic parameters, which may reflect subtle differences in the binding of these molecules in the active site. An earlier study used CD spectroscopy to investigate bovine milk galactosyltransferase and determined that only low amounts (10%) of \(\alpha\)-helix were present (26). A decrease in the amount of \(\alpha\)-helix was observed when UDP-galactose was added, but no change was observed, however, in the presence of UDP alone in contrast to our data obtained when UDP was added to GlcNAc-T V.

The CD spectra of mixtures of the enzyme and inhibitory substrate analog, which is also optically active, were fully accounted for by simple additivity of the individual spectra. Moreover, the substrate analog had no effect on the CD difference spectra of enzyme-substrate mixtures and the \(K_d\) derived from analysis of the spectral changes. These findings are consistent with an ordered mechanism in which the substrate UDP-GlcNAc first binds to GlcNAc-T V, and this complex then binds inhibitor. We cannot unequivocally rule out other models, particularly if the inhibitor binds to the enzyme and neither substrate with the active site appear to be dissimilar.

The results from the GlcNAc-T V kinetic assays demonstrate that the release of the catalytic products follows a sequential Bi-Bi mechanism. Therefore, the total reaction for the enzyme appears to be an ordered sequential Bi-Bi mechanism, which is similar to that proposed for GlcNAc-T II (28) but is distinct from that proposed for the \(\beta(1,4)\)-galactosyltransferase, which appears to function by a random mechanism (27). Kinetic data suggested that UDP-GlcNAc binds first to GlcNAc-T II and induces a conformational change to which the appropriate oligosaccharide acceptor could then bind (27); our results on UDP-GlcNAc binding to GlcNAc-T V are supportive of this model.

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