Differences between Asn-Xaa-Thr-Containing Peptides: A Comparison of Solution Conformation and Substrate Behavior with Oligosaccharyltransferase†

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ABSTRACT: A series of tripeptides that satisfy the -Asn-Xaa-Thr/Ser- primary sequence requirement [Marshall, R. D. (1972) *Annu. Rev. Biochem. 41*, 673-702] for N-glycosylation have been synthesized and examined as potential acceptors in an oligosaccharyltransferase assay. Of these, six (Ac-Asn-Ala-Thr-NH_2, Ac-Asn-Leu-Thr-NH_2, Ac-Asn-Asp-Thr-NH_2, Ac-Asn-D-Ala-Thr-NH_2, Ac-Asn-Pro-Thr-NH_2, and Ac-Asn-AIB-Thr-NH_2) were examined for solution conformational properties in dimethyl sulfoxide with use of amide proton temperature coefficients, $^3J_{HN\alpha}$ analysis [Pardi, A., et al. (1984) *J. Mol. Biol. 180*, 741-751], and 2-D ROESY experiments [Bothner-By, A. A., et al. (1984) *J. Am. Chem. Soc. 106*, 811-813]. The analysis reveals that the peptides that serve as acceptors in the transferase assay demonstrate similar conformational properties in solution. These are highlighted by a secondary structural motif that involves the interaction between the asparagine side-chain carboxamide and the backbone amide of the threonine. The peptides that show very poor acceptor, or even nonacceptor, properties in the oligosaccharyltransferase assay demonstrate different conformational features in solution. These observations may explain the distinct biological activity observed for these peptides.

Asparagine-linked glycosylation is a cotranslational process catalyzed by the membrane-associated enzyme oligosaccharyltransferase (Hubbard & Ivatt, 1981; Presper & Heath, 1985; Kaplan et al., 1987). This process, unique to eukaryotic systems, represents the primary metabolic step responsible for the formation of a covalent bond between oligosaccharide and polypeptide fragments and thus plays a central role in the cellular processes that depend on glycosylated proteins such as transport and intercellular recognition. Specifically, the enzyme mediates the cotranslational transfer of an oligosaccharide moiety from a dolichol-linked pyrophosphate donor to the primary amide nitrogen of an asparagine side chain within a nascent polypeptide chain.

It is now well-recognized that peptide conformation, in addition to steric and electronic effects, plays an important role in determining the interactions between short polypeptides and the macromolecular proteins with which they interact. This has been clearly demonstrated in investigations of the interactions between peptide hormones and neurotransmitters with receptors (Hruby & Mosberg, 1982) as well as in studies defining the interaction between peptides and the cAMP-dependent protein kinase (Bramson et al., 1987). Furthermore, it has been demonstrated that if a polypeptide is conformationally constrained to adopt the salient conformational features necessary for interaction with a protein, then affinity for the macromolecule may be enhanced and valuable information concerning its "bound" conformation can be deduced (Hruby & Mosberg, 1982).

With regard to asparagine-linked glycosylation, the minimum primary sequence requirements for all glycosyl acceptor substrates are well-defined. With few exceptions (Gavel & von Heijne, 1990), the glycosylation site is composed of an -Asn-Xaa-Ser/Thr- tripeptide sequence (Marshall, 1972; Struck & Lennarz, 1980), the structural elements of which are responsible for defining recognition of a specific asparagine residue and for augmenting the nucleophilic reactivity of the primary amide nitrogen. The middle amino acid in the sequence can be any of the naturally encoded primary amino acids, but the secondary imino acid proline is known to bestow nonacceptor properties on the peptide (Bause & Hettkamp, 1979). In addition, in synthetic tripeptides, which are also competent glycosylation substrates, it is necessary that the amino and carboxyl termini be masked in order for acceptor activity to be observed.

In terms of conformational requirements, the situation is far less definitive. Glycosyl transfer acceptor studies with synthetic peptides have been documented by Aubert et al. (1981), Bause et al. (1982), Bause (1983), Aubry et al. (1987), and Abbadi et al. (1989). In addition, structural studies involving circular dichroism (Aubert et al, 1981; Bause, 1983; Aubry et al., 1987) infrared and X-ray analyses (Aubry et al., 1987; Abbadi et al., 1989) have been carried out on peptides related to the acceptor sequence. In all cases, a clear indication emerges that the presence or absence of acceptor properties *could* be related to secondary structure forming tendencies in solution. Specifically, β-turn (Aubert et al., 1981), "loop" structures (Bause et al., 1982), and the Asx turn (Abbadi et al., 1989) have been proposed. However, at the present time, more structural information is needed to either consolidate one of the proposed conformational models or in fact determine if indeed solution conformational tendencies play any role in the glycosylation process.

Recently, a combination of one- and two-dimensional NMR[1] experiments has been utilized to evaluate conformational information from short peptides in solution (Gierasch et al., 1985; Dyson et al., 1988). These methods seem to be par-

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[1] Ac, acetyl; Bz, benzoyl; BOC, *tert*-butyl carbamate; NMR, nuclear magnetic resonance; COSY, two-dimensional correlated spectroscopy; NOE, nuclear Overhauser effect; CAMELSPIN, "cross-relaxation appropriate for minimolecules emulated by locked spins"; ROESY, rotating-frame two-dimensional nuclear Overhauser effect spectroscopy; AIB or B, α-aminoisobutyric acid; D-Ala or X, D-alanine. Standard one- and three-letter abbreviations are used for all natural amino acids.

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ticularly applicable to an investigation of the conformational tendencies of peptides that may be subject to asparagine-linked glycosylation in order to address the potential role that solution conformation may play in this process. Thus, we have undertaken an investigation of a series of tripeptides that meet the key requirements for an -Asn-Xaa-hydroxy amino acid recognition sequence within the substrate but differ principally in the nature of the central residue. This study includes an evaluation of the properties of each peptide as oligosaccharide acceptors in the transferase assay and a parallel examination of the solution conformational properties with use of amide proton temperature coefficients, $^3J_{HN\alpha}$ coupling constants (Pardi et al., 1984), and NOE studies. The information on through-space interactions is obtained through the use of a CAMELSPIN or 2-D ROESY NMR experiment (Bothner-By et al., 1984; Bax & Davis, 1985; Bax, 1988). These experiments have been performed in a variety of solvents (Gierasch et al., 1985; Dyson et al., 1988), and we have elected to carry out the spectroscopic studies in dimethyl sulfoxide. This polar, but aprotic, solvent was chosen since we considered it to represent the polar membrane interface where the nascent polypeptide substrate and the membrane-associated enzyme oligosaccharyltransferase are localized better than either water or a nonpolar organic solvent. Also, it has been reported that oligosaccharyltransferase-catalyzed glycosylation is significantly enhanced in the presence of nondenaturing levels (up to 13.5%) of dimethyl sulfoxide (Ronin & Aubert, 1982). This effect is thought to be due to an increase in the population (as observed by CD studies) of a peptide conformer recognized by the oligosaccharyltransferase.

Experimental Procedures

Peptide Synthesis. All peptides examined were synthesized by standard solution-phase peptide synthesis protocols with BOC α-amino-protecting groups and dicyclohexylcarbodiimide/1-hydroxybenzotriazole or *p*-nitrophenol ester mediated coupling reactions (Bodansky, 1984). The synthetic details and complete physical data for each peptide are described in supplementary material.

NMR Spectroscopy. All spectra were recorded on a Bruker WM-500 spectrometer operating at a proton resonance frequency of 500 MHz. Ac-Asn-Leu-Thr-NH_2, Ac-Asn-Ala-Thr-NH_2, Ac-Asn-Asp-Thr-NH_2, Ac-Asn-Pro-Thr-NH_2, Ac-Asn-AIB-Thr-NH_2 and Ac-Asn-D-Ala-Thr-NH_2 were pretreated by coevaporation with deuteriochloroform and subsequently dissolved in d_6-dimethyl sulfoxide (concentration ca. 4 mg/mL, 10 mM). Samples were placed in 5-mm NMR tubes (Wilmad Glass Co.).

NMR experiments were performed at ambient temperature (298 K) except if otherwise noted. Peak assignment was determined either in one-dimension (1-D) by homonuclear single-frequency decoupling experiments or through the use of a two-dimensional (2-D) ^{1}H COSY experiment. In the former case, the decoupler power used was maintained at the minimum required to instantaneously saturate the peak of interest. 2-D spectra were obtained in phase-sensitive mode and were mutiplied by a squared sine-bell apodization function prior to Fourier transformation. The spectral widths in both dimensions were typically 5000 Hz. NOE's were detected by use of 2-D spin-locked ROESY experiment, which is best suited to the detection of NOE effects in relatively small systems (MW 400–1500). These spectra were recorded at 299 K. In this experiment, the spin lock was achieved by applying a 200-ms pulse through the decoupler channel. The relaxation delay was 1.5 s. The transmitter offset was positioned in the center of the spectrum. The ROESY experiment conditions (namely, mixing time, radiofrequency power, and offset frequency) were varied in initial studies in order to verify that NOE connectivities observed represented proximity and did not in fact result from Hartmann–Hahn magnetization transfer (Cavanagh & Keeler, 1988; Dyson et al., 1988). The data matrix consisted of 256 t_1 increments containing 1K complex points. It should be noted that the ROESY cross-peaks, while useful in obtaining qualitative proximity information (<3 Å) cannot be easily used to derive quantitative distance information in the same way as NOESY data (Bauer et al., 1990).

The temperature dependence of the assigned amide-proton shifts was determined between 299 and 325 K. In all cases, the chemical shift was found to vary linearly with temperature. A minimum of six temperature steps were recorded in each experiment. Temperature calibration of the actual probe temperature was performed by use of an ethylene glycol standard.

Oligosaccharyltransferase Assay. The microsomal enzyme preparation utilized for the transferase assay was prepared from fresh pig liver. The preparation was based on a procedure described by Behrens and Tabora (1978) for rat liver. A 100-g sample of pig liver typically affords 2.5–5 mL of a microsomal preparation (~100 mg/mL protein) that can be stored at −85 °C in the presence of 30% glycerol. This material can be used for several months without significant loss of transferase activity. The transferase assay is based on that described by Sharma et al. (1981). In a typical assay 30000 dpm [^{3}H]-dolichylpyrophosphoryl-*N,N'*-diacetylchitobiose (specific activity 15–20 Ci/mmol) (Imperiali & Zimmerman, 1990) was dried down in an Eppendorf tube. To this was added peptide (dissolved in 10 μL of dimethyl sulfoxide) and 40 μL of assay buffer (50 mM Tris-HCl, pH 7.5; 1.2% Triton X-100; 10 mM $MnCl_2$). The reaction was then initiated with 150 μL of an enzyme solution (prepared by resuspending the microsomal fraction in 7.5 volumes of assay buffer). Reaction aliquots (3 × 60 μL) were removed at 2-min intervals and quenched into 1.2 mL of 3:2:1 chloroform/methanol/4 mM $MgCl_2$. The quenched reaction aliquots were treated as follows to isolate glycopeptide product away from unreacted lipid-linked donor. The upper aqueous phase was removed and the lower phase reextracted with two 0.6-mL portions of theoretical upper phase (12:192:186:2.69 chloroform/methanol/water/0.25 M $MgCl_2$). The combined upper phases were then counted (disintegrations per minute) in 5.5 mL of Ecolite (ICN). Nonacceptor peptides were evaluated for activity at a limiting concentration of 5 mM. Lack of any observable activity at this level led to a designation of a $K_M > 50$ mM. Acceptor peptides were evaluated at four concentrations; K_M's were determined from a $1/[S]$ vs $1/V$ plot.

The issue of whether the observed rates are indeed due to the enzyme-catalyzed glycosylation process, and not simply to hydrolysis of the lipid-linked substrate to afford a water-soluble disaccharide, has been addressed in the following experiments. First, reactions in the presence of all standard assay constituents initiated with thermally denatured microsomes show no discernible substrate hydrolysis. Second, the water-soluble radioactive material, obtained after the assay is run, has been prepared on a large scale and subjected to acid-catalyzed hydrolysis followed by gel filtration analysis on a Bio-Gel P-2 column (eluent 50 mM acetic acid). The major radioactive material eluted corresponds to a compound with higher molecular weight than *N,N'*-diacetylchitobiose.

Results

The 11 tripeptides examined in the oligosaccharyltransferase assay belong to two homologous series differing only in the

Table I: Enzyme Assay Results From Tripeptide Studies

peptide	K_M[a] (mM) X = Ac, Y = NH_2	K_M[a] (mM) X = Bz, Y = NHMe
X-Asn-Leu-Thr-Y	1.0	0.25
X-Asn-Ala-Thr-Y	2.0	ND[b]
X-Asn-Asp-Thr-Y	>50	3.3[c]
X-Asn-D-Ala-Thr-Y	>50	>30[d]
X-Asn-AIB-Thr-Y	>50	>30[d]
X-Asn-Pro-Thr-Y	>50	>50

[a] Similar V_{max} values were obtained for each peptide; 500 dpm/min ($\sim 5 \times 10^{-3}$) nmol min^{-1} mg^{-1} microsomal protein at a DolPPNAG-NAG concentration of 8.3 nM. [b] Not determined. [c] Y = NH_2 [d] Residual activity apparent at high peptide concentrations. K_M lies between 30 and 50 mM.

protecting groups used at the carboxyl and amino termini. Six of the tripeptides are blocked at the amino terminus by an acetyl group and at the carboxyl terminus by a primary amide, and five are blocked by a benzoyl group and a secondary amide. The rationale for investigating both series is as follows: First, it has been noted that when a peptide is a very poor acceptor (Rathod et al., 1986), use of a more hydrophobic amino-protecting group such as a benzoyl moiety increases the affinity of the enzyme for the substrate. Thus, by using the benzoyl-protected peptides, one is able to effectively increase the limits of detection of oligosaccharide acceptor properties in the assay. However, on the other hand, the spectroscopic studies proved to be more informative in the acetyl-protected series since the spectra of the benzoyl-protected peptides are complicated by overlapping of the benzoyl proton signals with the diagnostic amide resonances. By examination of both series of peptides, the maximum information concerning *both* acceptor properties and solution conformation can be derived. We have found that substitution of the benzoyl group for the acetyl does not appear to perturb the solution conformation in the 2-D experiment.

The K_M values for the peptides are shown in Table I. In cases where a direct comparison can be made between acetyl- and benzoyl-protected peptides (leucine or aspartic acid as the center residue), it can be seen that binding affinity is enhanced, as predicted, with the benzoyl group. Within the two series, only those peptides with L-amino acids at the center position show oligosaccharide acceptor properties. It is interesting to note that Bz-Asn-Asp-Thr-NH_2 is a substrate, albeit far poorer than the corresponding alanine- and leucine-containing peptides. This observation is contrary to original reports indicating that aspartic acid as the central residue cannot be tolerated in an oligosaccharide acceptor (Mononen & Karjalainen, 1984). The remaining tripeptides, with substitution of proline, D-alanine, or α-aminoisobutyric acid in the center position, are all either very poor acceptors or nonacceptors, regardless of the N-terminal protecting group.

Table II documents the amide proton temperature coefficients for the six acetyl-protected peptides. These values provide a measure of the amide proton solvent accessibility

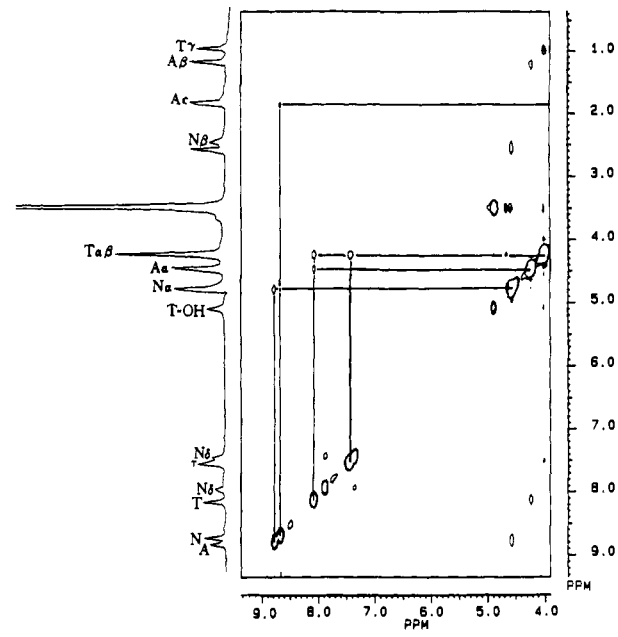

FIGURE 1: Portions of a 500-MHz ROESY spectrum of Ac-Asn-Ala-Thr-NH_2 at 299 K. The region shown encompasses the resonances of the backbone amide protons and the α-carbon protons. Cross-peaks arise from through-space (>3 Å) connectivities and can be assigned to the following interactions: $d_{\alpha N}$(Ac,Asn), $d_{\alpha N}$(Asn,Xaa), $d_{\alpha N}$(Xaa,Thr), $d_{\beta N}$(Thr,terminal), $d_{\alpha N}$(Thr,Thr), $d_{\beta N}$(Thr,Thr), and $d_{\alpha N}$(Asn,Asn).

and/or hydrogen bonding. In polar solvents, we have found that coefficients ranging from −4 to −7 ppb/K are typical of solvent-exposed protons, whereas those in the 0 to −3 ppb/K range are diagnositic of solvent shielding. [Values close to 0 ppb/K have been observed for conformationally constrained cyclic peptides in chloroform/dimethyl sulfoxide mixtures (Gierasch et al., 1985).]

With the exception of Ac-Asn-Pro-Thr-NH_2, the proton demonstrating the lowest temperature coefficient is that associated with the threonine amide. It is noteworthy that in similar studies with threonine-containing peptides that lack the proximal asparagine residue the temperature coefficient for the amide proton lies in the "normal" range for an unsequestered proton. Thus, we do not believe that this effect is residue-specific (Imperiali et al., unpublished results).

The $^3J_{HN\alpha}$ coupling constants for the amide protons within the tripeptide backbones are shown in Table III. The angular dependence of amide proton−αC coupling constants ($^3J_{HN\alpha}$) has been calibrated from protein structure information (Pardi et al., 1984). In general, small coupling constants (2–4 Hz) are observed when the ϕ dihedral angles are in the −30 to −60° range and larger values (7–10 Hz) result from ϕ values of −100 to −140°. The former angles are associated with either regular α-helix secondary structure or the ϕ dihedral of the $n + 1$ residue within a type I β-turn, while the latter are associated with extended β-sheet structure, random coil, or the ϕ dihedral

Table II: Temperature Dependence of Amide Proton Chemical Shifts ($-\Delta\delta/\Delta T$)[a] for Ac-Asn-Xaa-Thr-NH_2[b] in Dimethyl Sulfoxide[c]

	Asn	Asn δNH	Asn δNH	Xaa	Thr	terminal	terminal
Leu	4.5	4.1	5.4	4.8	2.7	3.7	3.7
Ala	5.0	4.5	5.7	5.3	3.1	4.0	4.0
Asp	4.6	3.8	4.8	2.7	1.5	2.9	4.6
D-Ala	5.0	4.5	3.9	7.6	3.0	5.5	5.4
AIB	6.1	5.2	6.4	7.2	3.1	5.0	3.3
Pro	5.5	3.8	2.1	n/a	4.0	6.0	6.0

[a] In parts per billion per Kelvin. [b] Xaa = Leu, Ala, Asp, D-Ala, AIB, Pro. [c] Primary amide proton signals were assigned by comparison with analogous peptides from the Bz-Asn-Xaa-Thr-NHMe series and from 2-D NMR data.

Table III: $^3J_{HN\alpha}$ Coupling Constants (in Hertz) for Ac-Asn-Xaa-Thr-NH_2[a]

	Asn	Xaa	Thr
Leu	7.9	8.4	8.4
Ala	7.0	7.7	8.3
Asp	6.4	7.3	8.4
D-Ala	6.9	7.6	8.7
AIB	7.0	n/a	8.6
Pro	7.8	n/a	9.1

[a] Xaa = Leu, Ala, Asp, D-Ala, AIB, Pro.

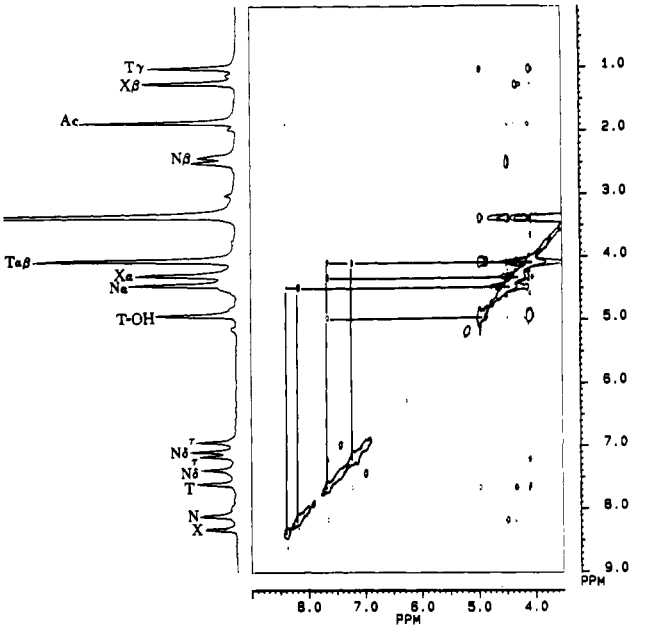

FIGURE 2: Portions of a 500-MHz ROESY spectrum of Ac-Asn-D-Ala-Thr-NH_2 at 299 K. D-Alanine residues are designated as X. The region shown encompasses the resonances of the backbone amide protons and the α-carbon protons. Cross-peaks arise from through-space (>3 Å) connectivities and can be assigned to the following interactions: $d_{\alpha N}$(Asn,D-Ala) (very weak), $d_{\alpha N}$(D-Ala,Thr), $d_{\alpha N}$(Thr,Thr), $d_{\beta N}$(Thr,Thr), and $d_{\alpha N}$(Asn,Asn) (strong).

of the $n + 2$ residue of a β-turn (Rose et al., 1985).

Figures 1–4 show segments of the ROESY spectra for four of the acetyl-protected peptides examined.

The 2-D ROESY spectrum of a representative peptidyl substrate, Ac-Asn-Ala-Thr-NH_2, is illustrated in Figure 1. (The corresponding spectra of Ac-Asn-Asp-Thr-NH_2 and Ac-Asn-Leu-Thr-NH_2 are not shown, but they feature strictly analogous cross-peaks.) All substrates are characterized by ROESY cross-peaks $d_{\alpha N}$(Ac,Asn),[2] $d_{\alpha N}$(Asn,Xaa), $d_{\alpha N}$(Xaa,Thr), $d_{\beta N}$(Thr,terminus), $d_{\alpha N}$(Thr,Thr), $d_{\beta N}$(Thr,Thr), and $d_{\alpha N}$(Asn,Asn), which are summarized graphically in Figure 5a.

The ROESY data for Ac-Asn-D-Ala-Thr-NH_2 are shown in Figure 2. D-Alanine substitution results in certain distinct changes in the cross-peak pattern. First, the strong backbone sequential connectivity $d_{\alpha N}$(Asn,Xaa) observed in the substrates is replaced by a very weak (relative to other signals within the same spectrum) cross-peak in Ac-Asn-D-Ala-Thr-NH_2. Instead, a strong intraresidue connectivity $d_{\alpha N}$(Asn,Asn), which was weak in the corresponding substrate spectrum, is noted. Also despite the change in chirality at the D-alanine αC, the sequential NOE $d_{\alpha N}$(Ala,Thr) is still present. This information is summarized in Figure 5b.

[2] The notation used to designate proton connectivities is fully described by Wuthrich et al. (1984).

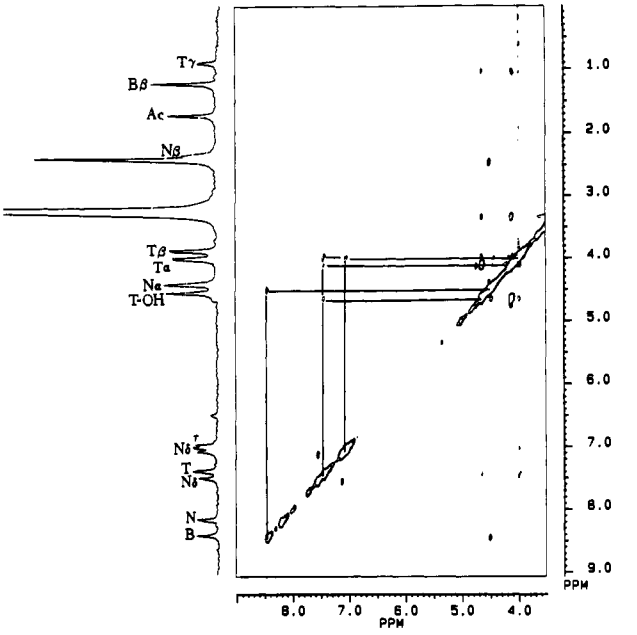

FIGURE 3: Portions of a 500-MHz ROESY spectrum of Ac-Asn-AIB-Thr-NH_2 at 299 K. The region shown encompasses the resonances of the backbone amide protons and the α-carbon protons. Cross-peaks arise from through-space (>3 Å) connectivities and can be assigned to the following interactions: $d_{\alpha N}$(Asn,AIB), $d_{\alpha N}$(Thr,Thr), $d_{\beta N}$(Thr,Thr), and $d_{\beta N}$(Thr,terminal).

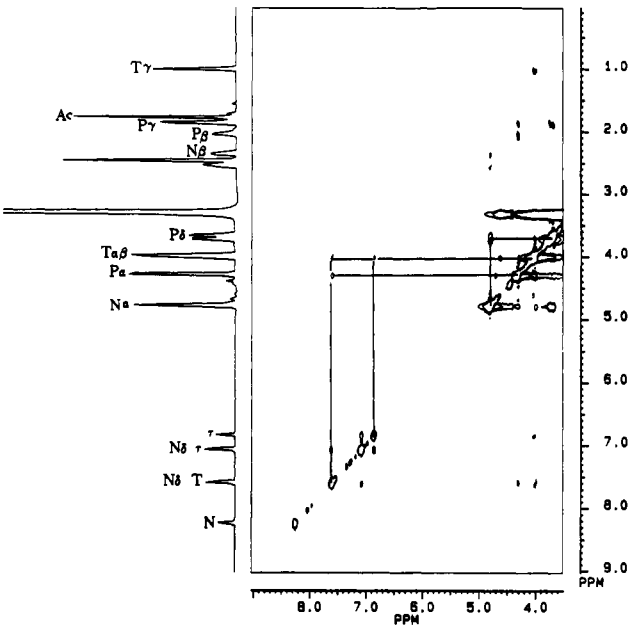

FIGURE 4: Portions of a 500-MHz ROESY spectrum of Ac-Asn-Pro-Thr-NH_2 at 299 K. The region shown encompasses the resonances of the backbone amide protons and the α-carbon protons. Cross-peaks arise from through-space (>3 Å) connectivities and can be assigned to the following interactions: $d_{\delta\alpha}$(Pro,Asn), $d_{\alpha N}$(Pro,Thr), $d_{\alpha N}$(Thr,Thr), $d_{\beta N}$(Thr,Thr), and $d_{\alpha N}$(Thr,terminal).

The ROESY spectrum for the peptide Ac-Asn-AIB-Thr-NH_2 is illustrated in Figure 3. The first notable feature of the spectrum is that the sequential and intraresidue interactions from the asparagine αCH are of very similar intensity. In general, however the information from this peptide is harder to interpret because the AIB αC is disubstituted, and therefore, no sequential information can be derived for the central residue. The key interpretation connectivities are illustrated in Figure 5c.

Figure 4 illustrates the ROESY spectrum for the nonacceptor peptide Ac-Asn-Pro-Thr-NH_2. The key cross-peaks

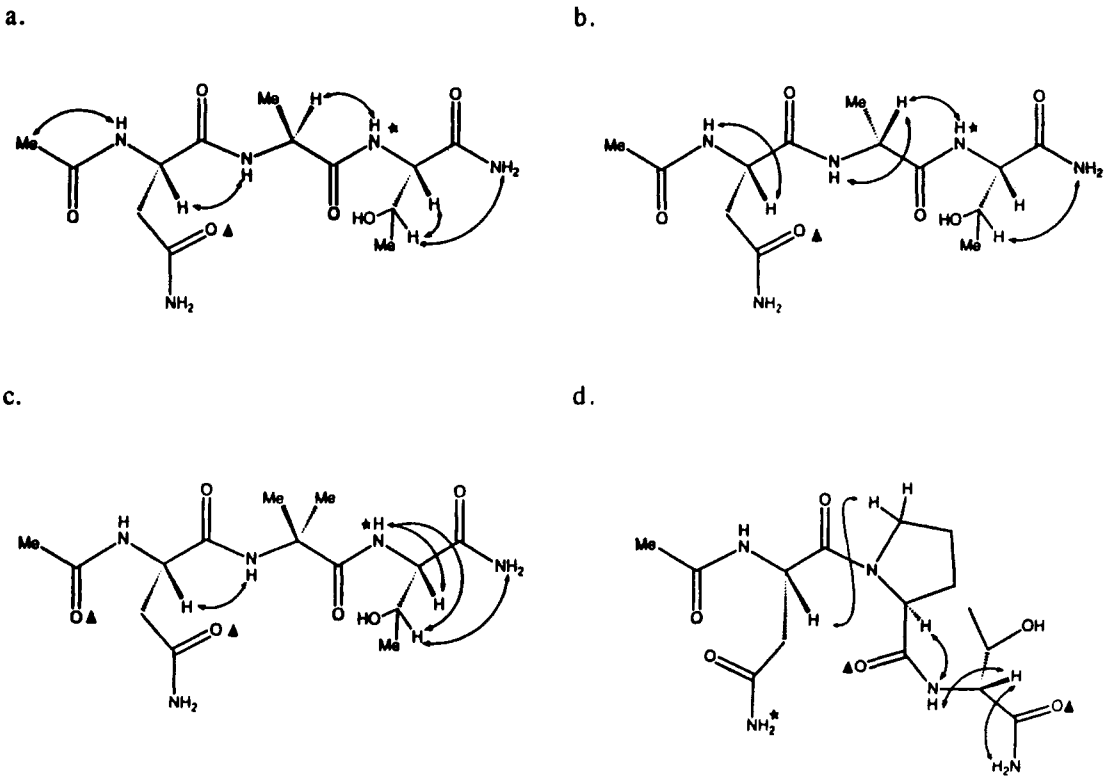

FIGURE 5: Graphical representations of connectivity and hydrogen-bonding information derived from spectroscopic studies of six acetyl-protected peptides. Key: ↔ NOE connectivity; ★ hydrogen-bond donor; ▲ hydrogen-bond acceptor. The structures shown are (a) Ac-Asn-Ala-Thr-NH_2, (b) Ac-Asn-D-Ala-Thr-NH_2, (c) Ac-Asn-AIB-Thr-NH_2, and (d) Ac-Asn-Pro-Thr-NH_2.

observed in the ROESY spectrum are $d_{\delta\alpha}$(Pro,Asn), $d_{\alpha N}$(Pro,Thr), and $d_{\alpha N}$(Thr,terminus). These are summarized in Figure 5d.

Discussion

It should be stated at the outset that in intrinsically flexible systems, such as short linear peptides, when comparing either through-space (NOE) or through-bond ($^3J_{HN\alpha}$) interactions, one is observing average information derived from an ensemble of conformations (Jardetzky, 1980) that, in the absence of intramolecular hydrogen bonds, are likely to be in rather random extended-chain conformations and hence provide little specific information. In the NMR analysis of the tripeptides, the purpose of the variable temperature studies is to pinpoint the presence of more ordered conformers that may be stabilized by intramolecular hydrogen bonding and hence exist in a finite population. Then, subsequent coupling constant and NOE studies can then be used to validate potential conformational models suggested by the variable temperature studies.

Conformational Analysis of Glycosyl Acceptor Tripeptides. The three tripeptide substrates examined appear to share common conformational characteristics. In the variable temperature data (Table II), it is significant that the threonine NH is shielded from the bulk medium as evidenced by the low temperature coefficients (−1.5 to −3.1 ppb/K) when compared with other values from the same peptide. In considering this observation, it is possible that this amide NH is shielded from the bulk medium by hydrogen bonding with an acceptor within the molecule. Due to ring size and amide constraints, only two carbonyl groups can be identified as likely acceptors in such an interaction. These are the N-terminal acyl carbonyl group or the asparagine side-chain primary amide carbonyl. If the former interaction were present, the $d_{\alpha N}$(Asn,Xaa) connectivity would be absent since this would place these protons on the opposite face of the molecule and hence out of connectivity range. However, with the latter interaction, the $d_{\alpha N}$(Asn,Xaa) would be expected to be strong. The other important connectivities observed, notably, the strong intraresidue interactions in the threonine residue, are compatible with a backbone to side chain H-bonding model. In addition, no other strong NOE connectivities are observed, which is in keeping with the above model. (Potential hydrogen-bond donor and acceptor pairs are also illustrated in Figure 5.)

Within the ROESY spectra of the acceptor peptides, we note the absence of interproton connectivities diagnostic of typical β-turn structures that generally include $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+2)$ interactions (Wuthrich et al., 1984). On the contrary, the substrate ROESY spectrum shows a series of five strong sequential connectivities between residues along the polypeptide backbone indicative of an extended conformation. Therefore, it is proposed that a consistent conformational model would incorporate a secondary structural motif resulting from asparagine side chain to peptide backbone interactions known as the Asx turn (Baker & Hubbard, 1984). In addition, the coupling constants for the amide protons within the peptide backbone are also indicative of an extended backbone conformation, since the presence of a typical β-turn conformation would be accompanied by a rather small $^3J_{HN\alpha}$ for asparagine (which would be the $n+1$ residue), which is not observed. Figure 6 shows a stereoview of this peptide conformation.

It is important to note that this conformational model is structurally consistent with the X-ray determination of Boc-Asn(δNMe)-Ala-Ser-NHMe carried out by Pichon-Pesme et al. (1988) and one of the conformational models suggested by Abbadi et al. (1989). Also, the analysis explains the kinetic results obtained by Bause et al. (1982) on glycosylation of linear and cyclic cysteine-containing peptides. Of the six peptides examined, Bause found that only one (Cys-Tyr-Asn-Cys-Thr-Ser-Val) failed to lose glycosyl acceptor potential on cyclization. This peptide *can* adopt the Asx turn motif in *both* oxidized and reduced forms.

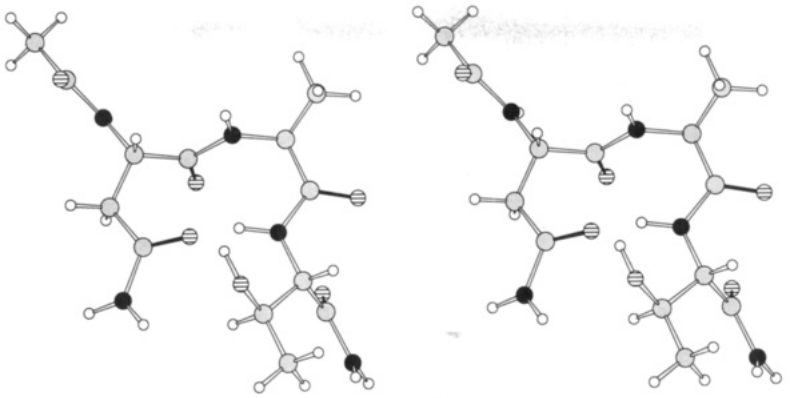

FIGURE 6: Stereoview of proposed conformational model for Ac-Asn-Ala-Thr-NH_2.

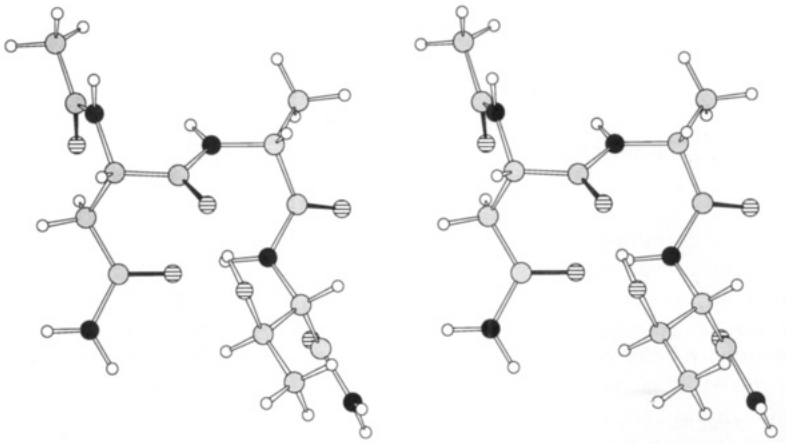

FIGURE 7: Stereoview of proposed conformational model for Ac-Asn-D-Ala-Thr-NH_2.

Conformational Analysis of Nonacceptor Tripeptides. An examination of the spectroscopic data for the three peptides that fail to serve as substrates in the glycosyl transfer assay reveals, in each case, that the peptide may adopt a conformation in solution that is distinct from that necessary for substrates.

In the D-alanine-containing peptide, while the variable temperature data also shows that the threonine amide is solvent shielded, the pattern of ROESY cross-peaks is quite distinct. A model consistent with these changes would still involve hydrogen bonding of the threonine NH to the asparagine side-chain amide carbonyl oxygen. However, in this case since the methyl group of the D-alanine residue would crowd the α-carbonyl group of the adjacent asparagine, the amide linking the two residues reorientates by 180°, thus relieving the steric interaction as illustrated in Figure 7. The change to this conformation is not unexpected from substitution of the central residue to one with opposite chirality.[3] Such a model would break up the sequential backbone interaction [$d_{\alpha N}$(Asn,Xaa)] and establish the strong intraresidue connectivity, $d_{\alpha N}$(Asn,-Asn), observed. In addition, this would place the D-alanine NH on the opposite face of the peptide, which might explain the observed increase in influence the temperature coefficient (now significantly higher at −7.6 vs −2.7 to −5.3 ppb/K for the substrates), since in this case NH is surrounded by different functional groups. The coupling constants observed for this peptide are consistent with this conformation.

[3] The Ramachadran plots of alanine vs D-alanine highlight the fact that the most accessible regions of conformational space are quite distinct for the two residues, with ϕ and ψ dihedral angles in the upper left-hand quadrant being most stable for alanine and in the lower right-hand quadrant for D-alanine.

The situation with the AIB-containing peptide cannot be compared directly with either of the examples described above, due to the previously mentioned loss of backbone information due to the disubstituted α-carbon within the peptide backbone. However, in the variable temperature data, two amide protons (the Thr NH and the terminus NH) show low-temperature coefficients and one amide (AIB NH) has a high-temperature coefficient rather analogous to the D-alanine-containing peptide and distinct from the substrate peptides. The profound effect that α-aminoisobutyric acid has on polypeptide conformational behavior has been reviewed by Prasad and Balaram (1984).

Finally, it has long been recognized that peptides containing proline as the central amino acid residue rarely (if at all) act as oligosaccharide acceptors in the transferase assay. Not surprisingly, both the ROESY (Figure 4) and variable temperature data testify to the fact that a central proline imparts very different conformational properties on the peptide in solution. The variable temperature data for this peptide are quite distinct. In this case, one of the asparagine side-chain amide protons shows the lowest temperature coefficient (−2.1 ppb/K), which implicates this proton in a H-bonding interaction. From examining conformational models, either the proline or threonine carbonyl groups could serve as potential H-bond acceptors. In either case, the key interaction between the threonine NH and the carbonyl of the asparagine side chain are broken. This may also explain why in this case the $^3J_{HN\alpha}$ value for threonine is somewhat higher (9.1 Hz).

CONCLUSIONS

The solution conformation of six peptides that incorporate possible N-glycosylation sites has been evaluated on the basis of amide temperature coefficients, $^3J_{HN\alpha}$, and 2-D ROESY NMR data. This study has been carried out in order to in-

vestigate if differences in solution conformation can explain the presence or absence of oligosaccharide acceptor properties in the glycosyltransferase assay. We have demonstrated that the three substrates examined share common conformational features in dimethyl sulfoxide solutions. Most importantly, the presence of an asparagine side chain to peptide backbone (the Thr NH) interaction, as in an Asx-turn (Baker et al., 1984), is proposed. This is also consistent with finding that certain functionalized amino acids play distinctive roles in protein secondary structure determination (Richardson, 1981). Examination of models shows that this interaction can serve to bring the threonine and asparagine side chains (crucial elements for glycosylation) into close proximity with each other and hence provide a mechanism whereby the functionality of one can influence the reactivity of the other. β-Turns have previously been implicated as the secondary structural motif responsible for bringing these functional groups together; however, these structural motifs do not appear to predominate in the solution studies that we have carried out. While the aspartate-containing peptides show similar conformational characteristics, the negatively charged side chain may be responsible for the diminished affinity (ca. 100-fold) for the oligosaccharyltransferase.

The three remaining peptides examined show very poor acceptor or nonacceptor properties. In all cases, it is possible that this diminished binding is due to a deleterious interaction at the enzyme binding site caused by the introduction of additional steric bulk. However, our study demonstrates that in each case alterations in the backbone conformation may perturb the finely tuned interactions between the side-chain functional groups in addition to the array of potential hydrogen-bonding interactions that can be made with the enzyme. Furthermore, binding to the enzyme is not able to overcome these inherent conformational tendencies.

These studies provide a working model for the rational design of glycosylation substrates. A key feature of this model suggests that conformation may play a role in determining substrate selection by oligosaccharyltransferase. Studies are currently in progress to further define this role through spectroscopic and kinetic evaluation of conformationally immobilized substrate analogues.

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Supplementary Material Available

Description of the solution-phase synthesis and spectroscopic characterization of each of the tripeptides examined in the paper (12 pages). Ordering information is given on any current masthead page.

Registry No. Asn, 70-47-3; Thr, 72-19-5; Ac-Asn-Leu-Thr-NH_2, 87980-75-4; Bz-Asn-Leu-Thr-NHMe, 102910-07-6; Ac-Asn-Ala-Thr-NH_2, 78939-41-0; Ac-Asn-Asp-Thr-NH_2, 132775-79-2; Bz-Asn-Asp-Thr-NHMe, 132775-83-8; Ac-Asn-(D-Ala)-Thr-NH_2, 132775-80-5; Bz-Asn-(D-Ala)-Thr-NHMe, 132775-84-9; Ac-Asn-AIB-Thr-NH_2, 132775-81-6; Bz-Asn-AIB-Thr-NHMe, 132775-85-0; Ac-Asn-Pro-Thr-NH_2, 132775-82-7; Bz-Asn-Pro-Thr-NHMe, 132775-86-1; oligosaccharyltransferase, 75302-32-8.

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