

Synthetic Substrates for Thyroid Oligosaccharide Transferase

Effects of Peptide Chain Length and Modifications in the -Asn-Xaa-Thr- Region

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The *N*-glycosylation of proteins is initiated by transfer of preassembled oligosaccharides from lipid carriers to an asparagine residue in the sequence Asn-Xaa-Ser(Thr). Various synthetic peptides were previously shown to act as effective acceptors when supplied to microsomal membranes. The present work was undertaken to delineate further within such peptides the structural determinants required for the *N*-glycosylation process. Several new peptides were synthesized to evaluate the effects of the chain length and of the modifications on the asparagine, Xaa or threonine residues: asparagine was replaced by glutamine, Xaa by proline or *N*-methylated alanine, threonine by an homoserine residue. Furthermore, both side chains of asparagine and threonine were simultaneously replaced by those of glutamine and homoserine respectively to restore a putative hydrogen bonding between the carbonyl and hydroxyl groups. The assays were performed with a solubilized form of the oligosaccharide transferase and the acceptor capacities of the peptides expressed by the ratio V/K_m of their apparent kinetic parameters V and K_m . Results showed that *N*-acetyl-tripeptide amides are excellent substrates for the enzyme. All modifications in the -Asn-Xaa-Thr- region resulted in a loss of acceptor capacity. The importance of the conformational aspect is discussed.

It is known from structural analysis of numerous glycoproteins that all N-linked oligosaccharide moieties are attached to the asparagine residue of Asn-Xaa-Ser(Thr) sequences [1]. These studies also showed that many of such asparagine residues were not glycosylated, so it was proposed that this tripeptide might be a necessary but not a sufficient requirement for glycosylation to occur *in vivo*. A number of laboratories have now clearly established that the initial steps in the assembly of asparagine-linked oligosaccharides consist in the synthesis of a common mannose-rich oligosaccharide, preformed as a lipid diphospho derivative and subsequently transferred as a whole to nascent protein chains (for a review, see [2]). Recent studies demonstrated the presence in microsomal membranes of hen oviduct [3], thyroid [4], rat liver [5] or yeast [6] of an oligosaccharide transferase activity which catalyses the transfer of an oligosaccharide from lipid carriers to well-defined exogenous Asn-Xaa-Ser(Thr)-containing acceptors: unfolded proteins or tryptic peptides [7], as well as synthetic peptides [4–6, 8–10] proved to be efficient substrates for the enzyme. The preliminary conclusion which could be drawn from these studies was that essentially no information on the primary structure other than the tripeptide sequence was necessary to ensure *N*-glycosylation *in vitro* [8, 9, 11]. Some discrepancies nevertheless remained concerning the minimal structural requirements for an Asn-Xaa-Ser-

(Thr)-containing sequence to be glycosylated: with the thyroid rough microsomal system, a peptide as small as the tripeptide H-Asn-Xaa-Thr-NH₂ was found to be an acceptor [8], whereas a similar one appeared to be ineffective with microsomal membranes from hen oviduct [9] or rat liver [10]. Addition at the asparagine amino group of either an amino acid [8–10] or an acetyl group [9] seemed to improve the acceptor capacity. In fact, all these data are not strictly comparable because the cell-free systems were different: the lipid donors were either mannose-labeled oligosaccharide lipids, exogenously supplied [4, 8] or endogenously generated in the assay [9], or else the much smaller dolichyl-diphospho-chitobiose [5, 6, 10]. Moreover, the authors used either a dispersing agent such as dimethylsulfoxide [4, 8], no detergent [9] or a non-ionic detergent [5, 6, 10].

Extending an earlier study on the structural features essential for a peptide to undergo glycosylation, this report presents assays of new synthetic peptides with a solubilized oligosaccharide transferase [12]. Furthermore, glucose-containing oligosaccharide lipids have been used since it appeared that the physiological lipid donors contain glucose [13].

MATERIALS AND METHODS

Materials

GDP-[³H]mannose (10.6 Ci/mmol) was from New England Nuclear Corp. All other chemicals were of analytical grade and obtained from commercial sources. Human calcitonin was a generous gift from Dr Argemi (CHU Timone, Marseille).

Abbreviations. Boc, *t*-butoxycarbonyl; (CH₂N)₂C, dicyclohexylcarbodiimide; HCONMe₂, *N,N'*-dimethylformamide; Me₂SO, dimethylsulfoxide; RNase, ribonuclease; N₂ph, diphenyl.

Nomenclature. Formulae of synthetic peptides have been written as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature for amino acid derivatives and peptides [Eur. J. Biochem. 27, 201–207 (1972)].

Table 1. Amino acid analyses of synthetic peptides I, and IV to XI

Values are means of three separate analyses after acid hydrolysis. Numbers in brackets refer to theoretical amount of each residue in the peptide

Amino acid	Residues in peptide								
	I	IV	V	VI	VII	VIII	IX	X	XI
Aspartic acid	0.99 (1)	1.00 (1)	1.00 (1)	0.97 (1)	0.96 (1)	1.03 (1)		1.01 (1)	
Serine						1.82 (2)	2.03 (2)	1.79 (2)	1.92 (2)
Threonine	0.95 (1)	1.00 (1)	0.98 (1)	0.99 (1)	0.93 (1)	0.94 (1)	0.96 (1)		
Glutamic acid	1.06 (1)			1.06 (1)	1.00 (1)	1.00 (1)	2.02 (2)	1.10 ^b (1)	2.13 ^b (2)
Proline				0.96 (1)					
Alanine	1.96 (2)	1.00 (1)			1.01 (1)				
Methionine						0.99 (1)	0.94 (1)	0.95 (1)	1.00 (1)
Leucine	0.96 (1)			1.01 (1)	0.95 (1)				
Tyrosine						0.95 (1)	0.81 (1)	0.98 (1)	0.82 (1)
Lysine			0.99 (1)						
Arginine	1.03 (1)			1.01 (1)	0.94 (1)				
Homoserine								— ^c	— ^c
N-Methyl alanine						— ^a			

^a N-Methyl-alanine yields faintly colored ninhydrin adducts so that it cannot be accurately measured.

^b Glutamic acid is contaminated by a by-product of the acid decomposition of homoserine.

^c Homoserine decomposes during acid hydrolysis, and so cannot be determined.

Synthetic Peptides

The following peptides were built by the solid-phase procedure [14] on commercially available (Beckman or UCB) benzhydrylamine-polystyrene/1% divinylbenzene resins: Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH₂ (I), N₂ph-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH₂ (II), Ac-Asn-Ala-Thr-NH₂ (III), H-Asn-Ala-Thr-NH₂ (IV), Ac-Asn-Lys-Thr-NH₂ (V), N₂ph-Ala-Leu-Glu-Asn-Pro-Thr-Arg-NH₂ (VI), H-Ala-Leu-Glu-Asn-MeAla-Thr-Arg-NH₂ (VII), H-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH₂ (VIII), H-Tyr-Gln-Ser-Gln-Ser-Thr-Met-NH₂ (IX), H-Tyr-Gln-Ser-Asn-Ser-Hse-Met-NH₂ (X) and H-Tyr-Gln-Ser-Gln-Ser-Hse-Met-NH₂ (XI). *t*-Butoxycarbonyl (Boc) protected amino acids (Bachem) were used throughout with the following masking groups for multifunctional residues: tosyl for guanidinium of arginine, benzyl for γ -COOH of glutamic acid, benzyl for the hydroxyls of homoserine, serine and threonine and 2,6-dichlorobenzyl for the phenol of tyrosine. Amino acid incorporations were drawn near completion by use of a double coupling procedure [twice 2.5 excess *t*-butoxy carbonyl-protected amino acid and 2.5 excess (CH₃N)₂C for 60 min] and checked by the ninhydrin test of Kaiser et al. [15]. For coupling of Boc-Asn and Boc-Gln, 1-hydroxybenzotriazole was added to prevent dehydration of ω -carboxamide group and the coupling was performed in HCONMe₂ [16]. The *t*-butoxycarbonyl group was cleaved by a 30-min treatment with 30% F₃CCOH/CH₂Cl₂ and subsequent neutralization was obtained by 5% diisopropylethylamine in CH₂Cl₂ (10 min). Acetylation on the resin by acetic anhydride in the presence of equimolar amount of diisopropylethylamine in HCONMe₂ was used either for blocking of remaining free amines in the case of a slightly positive ninhydrin test or for complete derivatization of the asparaginyl residue at the end of the synthesis of peptide V. Peptides were freed from the solid support and from protecting groups by reaction in anhydrous HF (60 min, 0°C) in the presence of 10% (v/v) anisole.

Purification of synthetic peptides was by a combination of gel filtration (Sephadex G-15 or Bio-Gel P₂) and ion-exchange chromatography on carboxymethyl-cellulose (CM52, Whatman).

Peptides II and VI were obtained by the action of 1-fluoro-2,4-dinitrobenzene onto the corresponding *N*²-free hepta-peptides, while peptide III was prepared by acetic anhydride treatment of the *N*²-free tripeptide. In all cases, modified peptides were recovered from reaction mixture after Bio-Gel P₂ filtration.

The regular and symmetrical shape of the elution pattern (absorbance at 230 nm) after CM-cellulose chromatography and lack of contaminating spots after high-voltage paper electrophoresis indicated homogeneity of the peptides.

Purity was also assessed by automatic amino acid analyses, on a Beckman 120°C, after acid hydrolyses (6 M HCl, 20 h at 110°C). Table 1 sums up analytical data.

Preparation

of Glucose-Containing [Man-³H]Oligosaccharide Lipids

Daily prepared thyroid rough microsomes (80 mg of protein) were incubated for 10 min at 37°C in presence of 0.5 μ M GDP-[³H]mannose (50 μ Ci) and 10 μ M UDP-glucose as described elsewhere [17]. Oligosaccharide lipids (8 $\cdot$ 10⁶ counts/min) were extracted as in [4]. The same batch was used in all assays of the present study.

Oligosaccharide Transferase Assay

The solubilization of the oligosaccharide transferase activity was achieved with 0.2% sodium deoxycholate, 0.5 M NaCl as described in [12]. The assay reactions contained the following components in a final volume of 150 μ l: 20 mM Tris/HCl, pH 7.5, 1 mM MnCl₂, 0.14 M sucrose, 10% Me₂SO, various concentrations of the potential peptide acceptor, glucose-containing [Man-³H]oligosaccharide lipids (2 $\cdot$ 10⁴ counts/min) dispersed in 0.2% sodium deoxycholate and 75 μ l of the deoxycholate extract (225–300 μ g of protein). After 30 min at 30°C, the reactions were stopped by adding 10 μ l of concentrated formic acid; the reaction mixtures were centrifuged at 11000 $\times$ g for 5 min. The [Man-³H]glycosylated peptides were isolated from the supernatant by high-voltage paper electrophoresis in 1.5 M formic acid as previously described [8]. The acceptor capacity of peptide III was evaluated either

Table 2. Comparative specificities of the membrane-bound and solubilized oligosaccharide transferase activities

The membrane-bound enzyme was assayed as in [8] using glucose-free [*Man*-¹⁴C]oligosaccharide-lipid donors whereas the solubilized activity was assayed with glucose-containing [*Man*-³H]oligosaccharide-lipids (see Materials and Methods). The rates of oligosaccharide transfer (in counts/min) to peptide VIII were arbitrarily taken as 100 to facilitate the comparisons

Peptide substrate		Oligosaccharide transferase activity					
		membrane-bound			solubilized		
		app K_m	relative rate of oligosaccharide transfer	relative acceptor efficiency	app K_m	relative rate of oligosaccharide transfer	relative acceptor efficiency
		μM			μM		
H-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH ₂	VIII	80	100	1.25	127	100	0.8
H-Ala-Leu-Gln-Asn-Ala-Thr-Arg-NH ₂	I	358	43	0.12	300	25	0.08
N ₂ ph-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH ₂	II	72	55	0.76	230 ^a	24 ^a	0.10 ^a
H-Asn-Ala-Thr-NH ₂	IV	2090	84	0.04	560	3	0.005
Ac-Asn-Ala-Thr-NH ₂	III	80	72	0.90	143	55	0.39

^a Single determination.

Table 3. Efficiencies of various synthetic peptides as substrates for the solubilized oligosaccharide transferase

K_m and V values are mean values of 3–5 independent experiments $\pm$ S.E. except for peptide II for which there was a single determination

Peptide	K_m	V	V/K_m
	μM	counts/min	
I. H-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH ₂	300 ($\pm$ 46)	1720 ($\pm$ 280)	5.7
II. N ₂ ph-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH ₂	230	1660	7.2
III. Ac-Asn-Ala-Thr-NH ₂	143 ($\pm$ 8)	3738 ($\pm$ 38)	26.1
IV. H-Asn-Ala-Thr-NH ₂	560 ($\pm$ 30)	185 ($\pm$ 98)	0.3
V. Ac-Asn-Lys-Thr-NH ₂	278 ($\pm$ 7)	5880 ($\pm$ 10)	21.2
VI. N ₂ ph-Ala-Leu-Glu-Asn-Pro-Thr-Arg-NH ₂	not acceptor		
VII. H-Ala-Leu-Glu-Asn-MeAla-Thr-Arg-NH ₂	not acceptor		
VIII. H-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH ₂	127 ($\pm$ 26)	6800 ($\pm$ 1780)	53.5
IX. H-Tyr-Gln-Ser-Gln-Ser-Thr-Met-NH ₂	not acceptor		
X. H-Tyr-Gln-Ser-Asn-Ser-Hse-Met-NH ₂	not acceptor		
XI. H-Tyr-Gln-Ser-Gln-Ser-Hse-Met-NH ₂	not acceptor		
XII. Human calcitonin	not acceptor		

by paper chromatography in ethylacetate/pyridine/acetic acid/water (5/5/1/3) or by electrophoresis in formic acid using a blank assay without acceptor as control.

RESULTS

Comparison of the Specificities of the Membrane-Bound and Solubilized Oligosaccharide Transferase Activities

As previously described for the rough microsomal enzyme [4, 8], the specificity of the solubilized oligosaccharide transferase towards its peptide substrate was assessed by studying the effect of increasing concentrations of a given synthetic peptide on the reaction velocity. The apparent kinetic parameters K_m and V were thus determined for a number of peptides and the ratio V/K_m was calculated in order to express a relative efficiency of the acceptor.

Table 2 shows the relative acceptor efficiencies of various peptides when using either the membranous or the solubilized form of the oligosaccharide transferase. Two different sizes of oligosaccharide lipids have been used, either containing glucose (solubilized activity) or not (membrane-bound activity). Using peptide VIII (residues 73–79 of porcine RNase)

a check was made that the apparent affinity of the enzyme and the rate of oligosaccharide transfer were not significantly modified by the glucose content of the lipid donors (data not shown). It can be seen in Table 2 that the results are roughly comparable whether the transferase is membrane-bound or solubilized: the best acceptor remains the RNase heptapeptide (peptide VIII), better than peptides I and II the sequence of which is derived from a tryptic fragment of human thyroglobulin. The addition of a dinitrophenyl group at the N terminus seems to be useful only when the transferase is membrane-bound, possibly because it facilitates the approach of the peptide to the enzyme, probably embedded in a lipophilic environment. It may be noticed also that, in both cases, peptide IV is the poorest substrate but turns out to be a good acceptor when acetylated (peptide III).

Acceptor Efficiency and Peptide Chain Length

Table 3 summarizes the apparent K_m and reaction velocity for the thyroglobulin peptide (I and II) and three shorter analogues (peptides III, IV, V). Fig. 1 shows the Lineweaver-Burk plots of the data. One of the main features is that the tripeptide Ac-Asn-Ala-Thr-NH₂ has a great capacity to be a

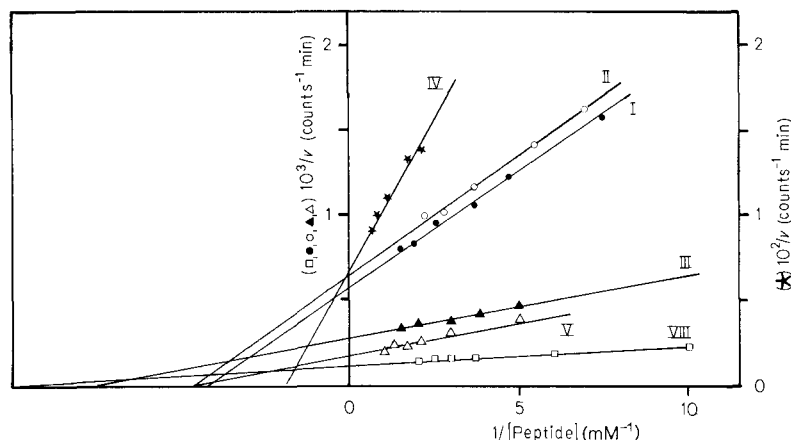


Fig. 1. Lineweaver-Burk plots of data from glycosylation assays (one typical experiment) with (□—□) peptide VIII, (●—●) peptide I, (○—○) peptide II, (★—★) peptide IV, (▲—▲) peptide III and (△—△) peptide V

substrate, even better than the heptapeptide of thyroglobulin (peptide I). A comparably high efficiency was also observed with the tripeptide Ac-Asn-Lys-Thr-NH₂ (peptide V), which differs only by the intermediate residue. One can notice (peptides III, V) the beneficial effect of the acetyl group at the N terminus of asparagine, an observation also made in an independent study recently reported [9]. The resulting ratio V/K_m was enhanced by a factor of 80. These findings strengthen the idea that all the primary structural requirements for a peptide to serve as substrate for the oligosaccharide transferase are comprised in the tripeptide sequence itself.

Human calcitonin is a natural polypeptide containing a vacant tripeptide sequence included in a disulfide loop: H-Cys-Ser-Asn-Leu-Ser-Thr-Cys- [18]. This polypeptide was unable to act as an acceptor for the transferase (Table 3). At first sight the presence of the disulfide bond, probably masking the tripeptide to the enzyme, hindered its glycosylation.

Structural Modifications in the Asn-Xaa-Thr Sequence

For a better understanding of the structural features recognized by the oligosaccharide transferase within the tripeptide sequence, each of the three amino acids was alternatively replaced by an analogue (Table 3), as follows.

The asparagine in position 4 of the RNase peptide was replaced by a glutamine residue, which corresponds to a shift of the carboxamide group to the γ position. The [Gln⁴] heptapeptide (peptide IX) was not an acceptor (Table 3), as recently also observed by others [5].

The presence of proline between the asparagine and threonine residues has already been reported to render the peptide unable to be glycosylated *in vitro* [5,8]. This conclusion was found again here with the use of the solubilized transferase (peptide VI in Table 3). Either the conformational constraint induced by the cyclic chain of proline or the lack of the amide proton may account for the loss of acceptor efficiency. Replacement of the intermediary alanine of peptide I by *N*-methyl alanine, which is believed to reduce the free rotation of the N-C α bond of the Xaa residue and to mimic the conformational features of proline, also yielded a derivative (peptide VII) unable to act as substrate for the oligosaccharide transferase.

An analogue of the reference RNase peptide was synthesized in which the threonine residue was replaced by a

homoserine residue (peptide X). In the [Hse⁶]heptapeptide, the hydroxyl group is shifted from the β to the γ carbon of the amino acid in position 6. This analogue was found to be devoid of acceptor capacity (see Table 3).

In another analogue (peptide XI) an attempt was also made to try to preserve the symmetric position of the carbonyl and hydroxyl side chain groups which were proposed by Marshall [1] to be hydrogen-bonded in the Asn-Xaa-Ser(Thr) sequence. The [Gln⁴,Hse⁶]heptapeptide was nevertheless unable to be glycosylated.

Despite the fact that all these modifications lead to peptides that could not act as substrates, it seemed possible that these analogues might be able to bind to the enzyme. To test this point, they were added separately in increasing amounts to an assay containing a saturating level of reference RNase peptide (peptide VIII). Neurotensin, which does not have an Asn-Xaa-Thr sequence, was used as control. The results of these competition studies are shown in Fig. 2. None of the analogue peptides except the [Pro⁵]peptide (peptide VI) was able to inhibit the glycosylation of the RNase peptide to an extent greater than neurotensin. Therefore the [Gln⁴]RNase [Hse⁶]RNase and [Gln⁴,Hse⁶]RNase peptides and the [Me-Ala⁵]thyroglobulin peptide seem unable to bind at the active site of the oligosaccharide transferase. In contrast, the [Pro⁵]peptide was found to interact with the binding site of the enzyme, as earlier deduced from its capacity to inhibit the glycosylation of the endogeneous proteins in the rough microsomal system [8].

DISCUSSION

In our previous work [4,8] and in studies from others [3,5–7,9,10], the source of oligosaccharide transferase was always the enzyme bound to more or less purified membranes. Although this approach was useful to indicate the potentiality of some typical peptide sequences to be glycosylated *in vitro*, the results may have been influenced by the penetration of the acceptors into the membranes, especially in the case of systems without any detergent or dispersing agent. The present report shows that, in thyroid, the apparent affinity of the solubilized oligosaccharide transferase towards the various peptides assayed is similar to that of the membrane-bound enzyme [8]. Therefore it is tentatively concluded that the active conformation of the transferase was the same

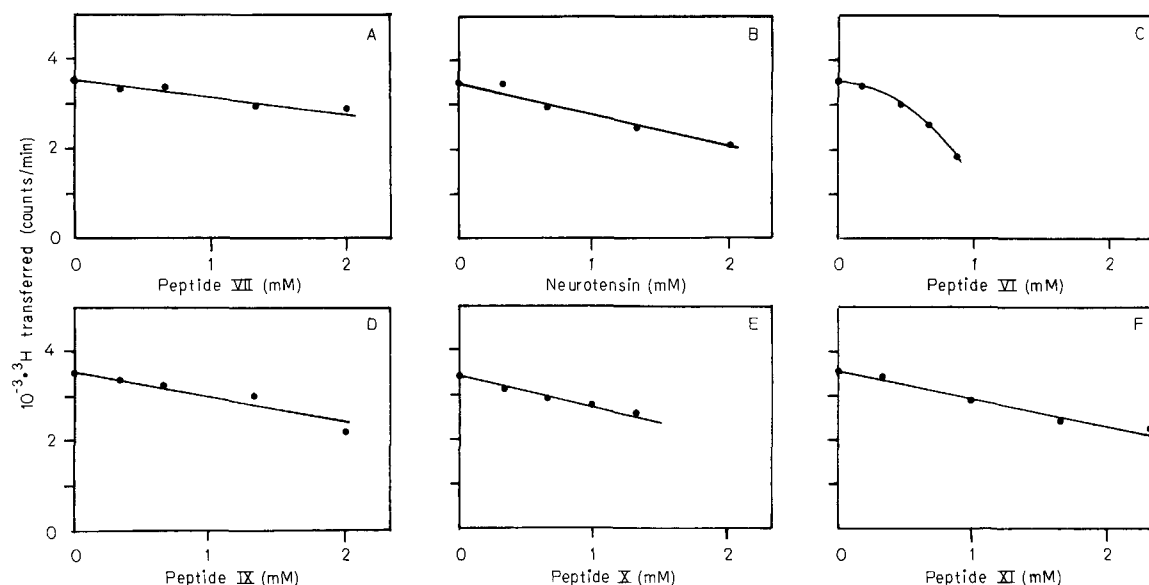


Fig. 2. Effect of non-acceptor peptides on the $[Man-^3H]$ oligosaccharide transfer to the reference RNase peptide ($333 \mu M$). (A) Peptide VII, (B) neurotensin, (C) peptide VI, (D) peptide IX, (E) peptide X, (F) peptide XI

in both cases and that it has been preserved during the detergent extraction. This observation is of interest for a further purification of the enzyme.

Concerning the lipid donors, it is worth mentioning that the present investigation was carried out for the first time with glucose-containing oligosaccharide lipids. The size and structural properties of these compounds have been studied elsewhere [17]. Since the specific radioactivity of a preparation of oligosaccharide lipids is presently difficult to assess and not easily reproduced from one batch to another, all the assays reported here were performed with the same preparation. It may be noticed that the efficiency of various peptides was not significantly affected by the presence of glucose in the lipid donors (Table 2). As recently underlined [12], the addition of 10% dimethylsulfoxide to the incubation mixture enhanced the yield of oligosaccharide transfer: after 30 min of incubation, approximately 40% of the supplied radioactivity was transferred to the reference RNase peptide (peptide VIII).

Altogether, our earlier findings have been confirmed in this study: the RNase heptapeptide (peptide VIII) is still an excellent acceptor, but several tripeptides also are good substrates for the oligosaccharide transferase. This provides further support to our first proposal [8, 11] that all the structural requirements for an asparagine residue to undergo glycosylation *in vitro* are located in the tripeptide sequence. Although never tested in our assay, peptides with a free carboxyl group close to threonine or serine seem to have a very low acceptor capacity [9, 10], something like that for peptides with a free α -amine at the asparagine [8–10]. But when the typical tripeptide sequence is included in between two amide bonds, as it is in proteins, it becomes a very good acceptor (peptides I–III, V, VIII). The smallest peptides acting as excellent substrates are of the type Ac-Asn-Xaa-Thr(Ser)-NH₂. This study shows also that amidation of the N terminus of asparagine (peptide III compared to peptide IV) leads mainly to an increase of the velocity of the oligosaccharide transfer. This and the result of another modification, dinitrophenylation, which was previously reported to inhibit *N*-

glycosylation in the microsomal system [8], suggest the possibility that the α -amine group of asparagine has to interact with the catalytic site of the enzyme once the substrate has been recognized. The α -NH group is able to do so when it is in the amide form more strongly than when it is in its ionized primary amine form, but this group would be non-reactive when dinitrophenylated, possibly because of a tight hydrogen bond with the *ortho* nitro group [19].

Changing the intermediate residue from alanine to lysine has no significant effect on the acceptor capacity of the peptide but the new positive charge enables the glycosylated peptide to be separated from the native one as well as from the neutral degradation products of oligosaccharide lipids by high-voltage paper electrophoresis. Therefore these tripeptides and especially Ac-Asn-Lys-Thr-NH₂ could be used as routine substrates for checking the oligosaccharide transferase activity.

It was known from the analysis of natural glycoproteins that the intermediate residue, Xaa, can be anyone of the 20 natural amino acids, except perhaps aspartic acid [20], meaning that its chemical nature is of no importance. It is surprising that even a proline residue has been detected in the Xaa position near a glycosylated asparagine in the 21–23 sequence region of horse ribonuclease [21]. In the assays *in vitro*, the presence of proline in the Xaa position has produced ineffective peptides as already reported by our laboratory [8] and by another one [5]. Confirmation of the negative influence of such an amino acid in this position was given by the lack of acceptor capacity of peptide VII, an analogue of peptide I in which alanine is replaced by *N*-methyl alanine. Here also, the α -amino group of the intermediate amino acid is a secondary amine but other features, namely conformational, in fact make the *N*-methyl-alanine structure very close to that of proline. However, unlike peptide VII, the [Pro⁵]peptide (VI) was able to inhibit partially the glycosylation of a reference peptide, suggesting thereby that it was probably recognized by the transferase.

Assays of several peptide analogues (peptides VIII–XI) have demonstrated the importance of the β -branching of

the two side chain functions that appear essential for the oligosaccharide transfer, i.e. the amide of the asparagine and hydroxyl of the serine residue. The modified peptides are not even recognized by the enzyme. β -Branching of the amide was expected to be important since no glutamine has ever been found glycosylated. Location of the hydroxyl group onto the β -carbon seems also of great importance, which could be explained by the putative hydrogen bonding proposed by Marshall [1]. When only the amide or the hydroxyl group is displaced to the γ position, this hydrogen bonding is not favoured; on the contrary, it was seen on a molecular model that some stabilizing conformation could be achieved when the two functions are simultaneously moved to the γ position. Unfortunately, the [Gln⁴,Hse⁶]peptide (peptide XI) was not a substrate and not even recognized by the enzyme. Steric requirements near the hydroxyl function may also have their importance: it was recently observed that in a cotranslational Krebs' II ascites tumor lysate system, β -hydroxynorvaline, which has side chain one carbon longer than that of threonine but has the hydroxyl in the same position, partially inhibited the *N*-glycosylation of nascent protein chains [22].

In conclusion, this study points out that the specificity of the oligosaccharide transferase towards its peptide acceptor is very narrow at the -Asn-Xaa-Ser(Thr)- region. A prerequisite for a peptide to be a substrate is probably a precise arrangement of most of the chemical groups situated in this tripeptide assemblage, i.e. this arrangement has to fulfill a specific spatial conformation. Any slight modification, such as the introduction of a methylene group on the side chain of asparagine or threonine or *N*-alkylation of the Xaa residue is sufficient to distort this basic structure and prevent *N*-glycosylation from occurring. The neighbouring amino acids before and after the tripeptide may of course contribute to the conformation of this region, affecting to different extents the efficiency of the transfer reaction. Such conformational effects may explain the variation of the reaction velocity observed in this study for different peptide substrates comprising the Asn-Xaa-Thr sequence (peptides I–III, V, VIII). A physico-chemical study on these peptides will have to be undertaken. Also, it is not known at the moment what influence the β -turn or loop structures, found by statistical calculations to be near most of the glycosylated asparagine residues in mature glycoproteins [23, 24], may have on the local conformation of those chemical groups involved in the interaction with the transferase.

The reason why, in a few *N*-glycoproteins, some asparaginyl residues of the chain are linked to carbohydrate moieties while some others, also included in typical Asn-Xaa-Thr/Ser sequences, remain unmodified is still not known. It is clear by now that, *in vivo*, the oligosaccharide transfer occurs in the early protein biosynthesis only while the nascent polypeptide is elongating [25, 26]. In the case of an unrestricted availability of the oligosaccharide transferase when the polypeptide chain is just emerging, glycosylation would then be restricted to the tripeptides that reached the favourable conformation under the influence of short-range interactions between amino acids. Alternatively, if the transfer reaction is the rate-limiting step in the biosynthetic pathway of *N*-

glycoproteins, then there is time for a more important folding of the protein and *N*-glycosylation would be limited to those tripeptides which are both accessible to the transferase and in a favourable conformational state.

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