PRIMARY STRUCTURAL REQUIREMENTS FOR *N*-GLYCOSYLATION OF PEPTIDES IN RAT LIVER

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1. Introduction

Microsomal preparations of thyroidea [1], rat liver [2] and yeast [3] are capable of synthesizing *N*-glycosidic linkages by transferring dolichyldiphosphate-activated saccharides to an asparaginyl residue of various synthetic peptides. All potent acceptor peptides contained the triplet sequence Asn–X–Thr/Ser, which has been predicted to be a necessary prerequisite for *N*-glycosylation [4]. Changing this 'marker sequence' either by replacing asparagine or the hydroxy amino acid results in a complete loss of acceptor capability of the corresponding peptide [2].

Extending our studies on primary structural requirements for *N*-glycosylation, we investigated systematically the influence of different parameters on the glycosyl acceptor properties of peptides, using the following reaction as a standard enzyme test:

$$\text{Dol-P-P}-[^{14}\text{C}]\text{GlcNAc}_2 + \text{Asn}-\text{peptide} \xrightarrow[N\text{-glycosyltransferase}]{Mn^{2+},\ \text{Triton X-100}} \underset{\displaystyle \text{GlcNAc}_2}{\text{Asn}-\text{peptide}} + \text{Dol-P-P}$$

It will be shown that, besides the Asn–X–Thr/Ser sequence *N*-glycosylation is also affected by the chain-length of acceptor peptides as well as by the type of the amino acid X in the 'marker sequence'.

Abbreviations: Dol-P, Dol-P-P, dolichyl mono(di)phosphate; GlcNAc, *N*-acetylglucosamine; GlcNAc, di-*N*-acetylchitobiose; boc-, *t*-butoxycarbonyl-; DNP-, 2,4-dinitrophenyl-; DNS-, 1-dimethylaminonaphthalene-5-sulfonyl-

2. Materials and methods

2.1. *Materials*

UDP-*N*-acetyl-[^{14}C]glucosamine (323 Ci/mol) was obtained from the Radiochemical Centre, Amersham. Dol-P and UDP-*N*-acetylglucosamine were from Sigma. Bio-Beads S-X 1, chloromethylated, were purchased from Bio-Rad, boc-amino acids from Serva. 'Protosol' was from NEN. All other chemicals were from commercial sources in the highest purity available.

2.2. *General methods*

Rat liver microsomes were prepared as in [5]. Peptides were synthesized by the solid-phase method according to [6,7]. Dinitrophenylation and dansylation were done as in [3]. Protein was determined by the Lowry method [8]. Radioactivity was detected either by radioautography with Sakura QH X-Ray Film or measured in a liquid scintillation counter (Delta 300, Searle Analytic Inc.).

2.3. *Preparation and purification of Dol-P-P-di-N-acetyl-[^{14}C]chitobiose*

Crude pig liver microsomes (400 μg) were incubated at room temperature in 100 μl total vol. containing 20 μg exogenous Dol-P, 50 mM Tris–HCl (pH 7.5), 0.8% Triton X-100, 10 mM $MnCl_2$, 4 mM GTP and 0.5 μCi UDP-[^{14}C]GlcNAc (323 Ci/mol). After 30 min, 50 μg unlabeled UDP-GlcNAc were added

and the incubation continued for a further 30 min. The reaction was terminated by the addition of 1 ml methanol and the glycolipid isolated by partition as in [9]. Further purification was done on DEAE-cellulose according to [10] and Silica gel chromatography with $CHCl_3/CH_3OH$/1 M NH_4OH (65/25/4, by vol.) as solvent. The resulting [^{14}C]glycolipid was chromatographically homogeneous and used for the standard enzyme test. Its specific activity was not determined.

2.4. *Standard assay for measuring* N-*glycosyltransferase activity*

The incubation mixtures contained in 50 μl final vol.: Dol-P-P-[^{14}C]$GlcNAc_2$ as indicated, 50 mM Tris–HCl (pH 7.5), 0.8% Triton X-100, 10 mM $MnCl_2$, 0–2.0 mM exogenous peptide and 100–300 μg microsomal protein. Incubations were run at room temperature and stopped by adding 0.5 ml methanol at chosen times. Samples were centrifuged, the supernatants removed and analyzed either by paper electrophoresis or partition chromatography as in [3]. Glycosylation of endogenous protein was measured according to [2].

3. Results and discussion

3.1. N-*glycosylation as a function of chainlength and hydrophobicity of peptides*

The results summarized in table 1 demonstrate that the minimum chainlength of a pentapeptide is required for *N*-glycosylation to occur. Neither tripeptide VII nor tetrapeptides IV and VI function as glycosyl acceptors. It is important to note that both N- and C-terminal elongation of the 'marker sequence' by at least one amino acid is necessary to give a peptide substrate with reasonable glycosyl acceptor properties (III). A further C-terminal extension of pentapeptide III by valine increases glycosylation rate ~5-fold, while N-terminal elongation of hexapeptide II brings about no significant improvement (I).

Peptides with free N-terminal asparagine are not glycosylated. This observation agrees with recent findings in yeast [3] and oviduct [11]. Obviously a free amino group on the asparagine residue is not tolerated by the *N*-glycosyltransferase. It is interesting that the structure of the blocking group appears to be of no importance, as can be seen from the similar acceptor properties of peptides III and XI, which are

Table 1
Glycosyl acceptor efficiency in dependence on chainlength and hydrophobicity of peptides

	Amino acid sequence	Conc. (mM)	^{14}C incorporation into			
			Peptide		Endogenous protein	
			(cpm)	(%)	(cpm)	(%)
Control experiment		–	–	–	950	23.8
I	Glu–Lys–Tyr–Asn–Leu–Thr–Ser–Val	1.15	1760	44.0	510	12.8
II	Tyr–Asn–Leu–Thr–Ser–Val	1.15	1690	42.3	500	12.5
III	Tyr–Asn–Leu–Thr–Ser	1.15	310	7.8	760	18.8
IV	Tyr–Asn–Leu–Thr	1.15	<40	<1	870	21.8
V	Asn–Leu–Thr–Ser–Val	1.15	75	1.9	840	21.0
VI	Asn–Leu–Thr–Ser	1.15	<40	<1	810	20.3
VII	Asn–Leu–Thr	1.15	<40	<1	900	22.5
VIII	Tyr–Asn–Leu–Thr–Ser–NH_2	1.15	980	24.3	640	16.0
IX	Tyr–Asn–Leu–Thr–NH_2	1.15	<40	<1	880	22.0
X	Asn–Ala–Thr–Val	1.15	<40	<1	920	23.0
XI	DNP–Asn–Ala–Thr–Val	1.15	250	6.3	880	22.0
XII	DNS–Glu–Lys–Tyr–Asn–Leu–Thr–Ser–Val	1.15	1320	33.0	560	14.0
XIII	DNS–Tyr–Asn–Leu–Thr–Ser–Val	1.15	1880	47.0	440	11.0

Experimental conditions were as in section 2; Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose, 4000 cpm (100%); incubation 30 min

in contrast to the resistance to glycosylation of VI and X.

Charge effects at the C-terminal of the 'marker sequence' might also be responsible for the lack of acceptor capability of peptide IV and the relative low glycosylation rate of III. To resolve this question we converted III and IV into their amide derivatives VIII and IX. Sugar transfer to the amides should be improved and become comparable to peptides II and III, if the negative charge at the terminal α-carboxyl group supplies the main reason for the poor or lacking acceptor properties of III and IV. Indeed, glycosyl transfer to the amide VIII is found to be ~3-times faster than to III, but still ~2-times slower in comparison to the hexapeptide II. This suggests that, in spite of the relatively long distance from the asparaginyl sidechain the amino acid residue itself has a distinct influence on the acceptor capability besides the aforementioned charge effects. This observation is strongly supported by the observation that neither peptide IV nor its amide derivative IX are glycosylated, signifying an absolute requirement of an amino acid residue next to the hydroxy amino acid of the 'marker sequence'. From these results it appears that the nature of the C-terminal region of the 'marker sequence' has a greater influence on the acceptor properties than the N-terminal side.

The hydrophobic nature of the sugar intermediates which are involved in the glycosylation process, and the necessity of detergents for the catalytic activity of *N*-glycosyltransferase suggest that hydrophobic residues in the peptide substrates may, to a certain degree, contribute to their binding to the enzyme and thereby influence the glycosylation rate. In order to introduce a hydrophobic group we dansylated two of the peptides and tested their acceptor capabilities. Indeed, XIII shows a slightly higher glycosylation rate than the free peptide, which may support the above hypothesis. The considerably reduced acceptor properties of peptide XII, however, which we found to be reproducible, point to a much more complex situation.

In contrast to our observations, the successful glycosylation of Asn–Ala–Thr–NH_2 in the thyroid system has been described [1], but a total loss of acceptor activity was observed after dinitrophenylation of this tripeptide. Apparently the *N*-glycosyltransferase from thyroidea possesses a different specificity with respect to the peptide substrates than the transferase from rat liver.

3.2. *Influence of the structure of X in the triplet sequence Asn–X–Thr on the rate of* N*-glycosylation*

In order to elucidate the role of an amino acid exchange in X, we synthesized a series of hexapeptides derived from Tyr–Asn–X–Thr–Ser–Val, in which X stands for glycine, leucine, lysine, aspartic acid and proline. Glycosyl transfer rates to these various peptides are summarized in table 2. Strikingly, 3 peptides, those containing glycine, leucine and lysine, are glycosylated to about the same extent. This indicates that,

Table 2
Influence of the amino acid X in the triplet sequence Asn–X–Thr on glycosyl acceptor properties of peptides

Amino acid sequence		Conc. (mM)	^{14}C incorporation into			
			Peptide		Endogenous protein	
			(cpm)	(%)	(cpm)	(%)
Control experiment		–	–	–	550	14.0
XIV	Tyr–Asn–Gly–Thr–Ser–Val	1.1	1250	32.5	230	6.0
XV	Tyr–Asn–Leu–Thr–Ser–Val	1.1	1300	33.0	200	5.0
XVI	Tyr–Asn–Lys–Thr–Ser–Val	1.1	1400	36.0	210	5.5
XVII	Tyr–Asn–Asp–Thr–Ser–Val	1.1	300	7.5	250	6.5
XVIII	Tyr–Asn–Pro–Thr–Ser–Val	1.1	no acceptor		500	12.5

Experimental conditions were as in section 2; Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose, 3900 cpm (100%); incubation 10 min

at least in vitro, small size (glycine) as well as hydrophobicity (leucine) or a positive charge (lysine) are of no significant consequence for the acceptor capability. Substitution of X for aspartic acid, however, results in an ~5-fold decrease in glycosyl transfer. Probably the accumulation of negative charges at the active site of the *N*-glycosyltransferase, represented by the β-carboxyl group of aspartic acid and the phosphates of the glycosyl donor Dol-P-P-$GlcNAc_2$ renders simultaneous binding of both substrates more difficult. In agreement with this interpretation is the observation that in the amino acid sequences of ~30 glycoproteins glycine, lysine and leucine occur at the position X of the 'marker sequence' with normal frequency, whereas no aspartic acid is found in this position.

The proline peptide XVIII is not an acceptor despite the essential triplet sequence Asn–X–Thr [1,2]. This result documents the significance of a specific interaction between asparagine and the hydroxy amino acid, which is necessary for the catalytic action of the *N*-glycosyltransferase and which is abolished by the particular structure of this imino acid.

3.3. *Glycosylation of endogenous protein acceptors*

We found an inverse relationship between the rate of glycosyl transfer to peptides and endogenous acceptors, which probably originates from a competition at the active site of the *N*-glycosyltransferase (see table 1, 2). The lack of inhibition of endogenous glycosylation by the shorter non-acceptor peptides indicates that these substrates are not bound to the enzyme in spite of the presence of the 'marker sequence'. Nevertheless, we recently demonstrated that non-acceptor hexapeptides, derived from Tyr–Asn–Leu–Thr–Ser–Val by an exchange of either asparagine or threonine showed a small, but reproducible inhibition of endogenous glycosylation [2]. From these data one can conclude that binding and glycosyl transfer require both the triplet sequence and a minimum chainlength of peptide acceptors.

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