STUDIES ON THE ACCEPTOR SPECIFICITY OF ASPARAGINE-*N*-GLYCOSYL-TRANSFERASE FROM RAT LIVER

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1. Introduction

On the basis of an examination of the amino acid sequence of numerous glycoproteins Marshall proposed that the tripeptide sequence Asn—X—Thr/Ser is a necessary, but not sufficient prerequisite for *N*-glycosylation in vivo [1]. Recently it has been demonstrated that glycosyltransferases from oviduct catalyze the transfer of Dol-P-P-bound oligosaccharides into denatured exogenous proteins as well as into smaller peptides derived from these [2]. All substrates tested as glycosyl acceptors contained the tripeptide sequence mentioned above, as did a series of synthetic peptides, which have been shown to function partially as good acceptors for *N*-glycosylation in vitro in the thyroid system [3] and in yeast [4].

In order to get more detailed information on the specificity of *N*-glycosyltransferases and to elucidate the structural requirements for *N*-glycosylation, a series of hexapeptides has been synthesized and tested as glycosyl acceptors, using microsomal fractions of rat liver as enzyme source and Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose as glycosyl donor. This substrate contains the smallest homogeneous sugar unit, which has been shown to be transferred to endogenous protein acceptors [5]. The advantages of this well-defined glycosyl donor compared with Dol-P-P-oligosaccharides, which are used by other authors, are obvious.

Abbreviations: Dol-P-P, dolichyl diphosphate; boc-, *t*-butoxycarbonyl-

2. Materials and methods

2.1. *Materials*

Bio-Beads S-X 1, chloromethylated were obtained from Bio-Rad, boc amino acids from Serva, Heidelberg. 'Protosol' was from NEN. All other chemicals were purchased from commercial sources. Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose (spec. act. 300 Ci/mol) was provided by Mrs U. Bergner and Dr L. Lehle, University of Regensburg.

2.2. *General methods*

Microsomal fractions from rat liver were prepared as described in [6]. Protein was determined by the Lowry method [7]. β-Elimination and hydrazinolysis were done as described in [8,9]. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter.

2.3. *Peptide synthesis*

Peptides were prepared by the solid phase method according to [10,11]. Purification was done by chromatography on Sephadex G-15 in 0.5 M acetic acid. Purity of peptides was checked by amino acid analysis and thin-layer chromatography on Silica gel plates with *n*-butanol/acetic acid/water (4/1/1) as solvent system.

2.4. *Assay for measuring N-glycosyltransferase activity*

Labeled Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose ($4–8 \times 10^3$ cpm) was dried under nitrogen and dispersed in 50 mM Tris/HCl (pH 7.4) containing 1.2% Triton X-100, 10 mM $MnCl_2$, 100–200 μg microsomal

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protein and 0–2 mM exogenous peptide in 50 μl total vol. After incubation at room temperature for 0–60 min reactions were terminated by adding 200 μl methanol. Samples were centrifuged and the supernatants, which contained the [^{14}C]glycopeptides, were separated and analyzed by electrophoresis in 1.5 M formic acid on cellulose plates or Whatman 3 MM paper. For measuring ^{14}C transfer to endogenous protein, pellets were washed repeatedly with chloroform/methanol (2/1) to remove unreacted Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose. The residual material was solubilized by adding 200 μl Protosol and radioactivity counted.

3. Results and discussion

3.1. *Transfer of di-N-acetyl-[^{14}C]chitobiose to Tyr–Asn–Leu–Thr–Ser–Val peptide*

Microsomal fractions of rat liver, incubated with Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose and the synthetic hexapeptide Tyr–Asn–Leu–Thr–Ser–Val, incorporate radioactivity rapidly and efficiently into a labeled compound, which is found only in the presence of the exogenous peptide. Its lower electrophoretic mobility in 1.5 M formic acid indicates a higher molecular weight as a result of sugar transfer. In several incubations with different microsomal preparations yields of 65–75%, based on added glycosyl donor, have been found under standard conditions (60 min; 1.4 mM peptide). Figure 1 shows the time-dependence of ^{14}C incorporation into the hexapeptide. The initial linear transfer slows down after 30 min because of substrate depletion and reaches a plateau at 65–70% incorporation. Saturation kinetics are observed for the ^{14}C transfer in dependence on the concentration of the acceptor peptide (fig.2). The app. K_m value taken from the graph is 0.29 mM and stresses the good acceptor efficiency of this peptide substrate.

The formation of a *N*-glycosidic linkage between asparagine and di-*N*-acetyl-[^{14}C]chitobiose can already be assumed from the use of a lipidpyrophosphate bound sugar derivative as glycosyl donor [5,12]. Under conditions of β-elimination, a standard procedure known to break *O*-glycosidic bonds to serine or threonine [8], no ^{14}C material was released, thus excluding an *O*-glycosidic binding of the sugar residue. *N*-glycosylation is further supported by the observation that hydrazinolysis of the [^{14}C]glycopeptide leads to a labeled compound, which was chromatographically identified as chitobiose.

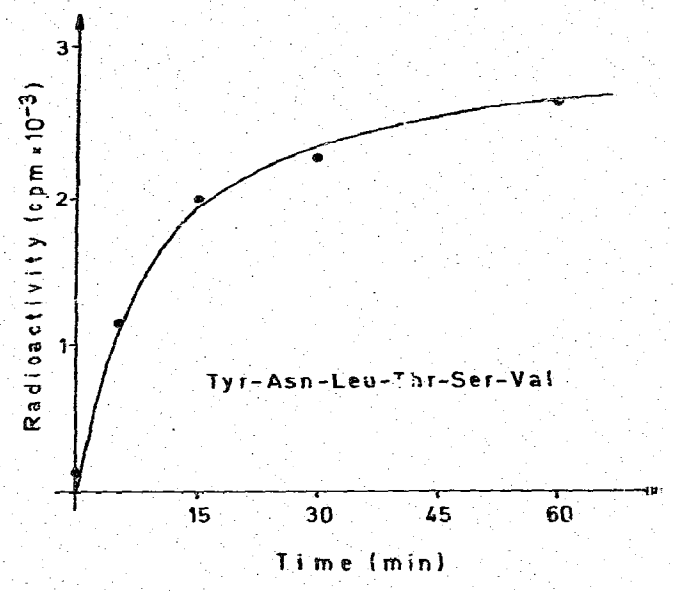

Fig.1. Time course of ^{14}C incorporation into acceptor peptide (peptide 1.4 mM, Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose 3.900 cpm).

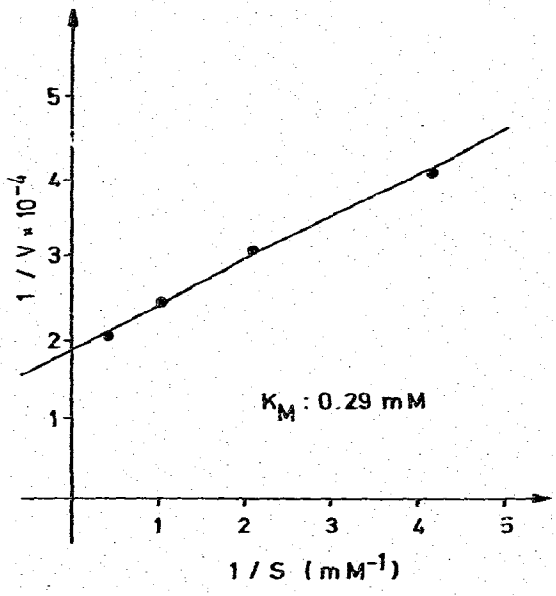

Fig.2. Di-*N*-acetyl-[^{14}C]chitobiose transfer as a function of peptide concentration. Ordinate: reciprocal incorporation of radioactivity (cpm) into peptide fraction after 15 min incubation time.

3.2. *Peptide substrates with modified 'marker sequence'*

Table 1 summarizes the results obtained with a series of hexapeptides, which differ only by the exchange of one amino acid in the tripeptide sequence Asn–Leu–Thr. These peptides had been synthesized in order to reduce changes in acceptor specificity to this one amino acid exchange. The results in table 1 point out that only the Tyr–Asn–Leu–Thr–Ser–Val peptide serves as glycosyl acceptor. Replacing asparagine by aspartic acid and glutamine, respectively, or threonine by valine cancels acceptor activity completely. This can be considered as a proof that for *N*-glycosylation, at least with Dol-P-P-activated sugars, the tripeptide sequence Asn–X–hydroxyamino acid is essential. Until now it has not been possible to obtain such evidence because of lacking peptide substrates directly comparable with each other.

It is striking that the Tyr–Asn–Pro–Thr–Ser–Val peptide is no acceptor despite the Asn–X–Thr sequence. The reason may be ascribed to the particular structure of the proline residue, by which an interaction between asparagine and threonine possibly necessary for the substrate binding or the catalytic action of the *N*-glycosyltransferase is abolished.

As could be expected, the excellent acceptor properties of the Tyr–Asn–Leu–Thr–Ser–Val peptide result in a nearly complete inhibition of the ^{14}C transfer to endogenous membrane protein. All non-acceptor peptides, except the one containing proline, also show a small, but reproducible inhibition of endogenous protein glycosylation as well as of ^{14}C incorporation into the Asn–Leu–Thr peptide (data not shown), possibly originating from a competition at the binding site of the *N*-glycosyltransferase.

4. Conclusions

The following conclusions may be drawn:

1. The Asn–X–Thr/Ser sequence is a necessary and sufficient condition for *N*-glycosylation in vitro, provided an additional amino acid is bound to the N-terminus [4]. Replacement of asparagine or the hydroxyamino acid causes a complete loss of acceptor activity.
2. Peptides with an Asn–Pro–Thr/Ser sequence cannot be glycosylated, indicating that a necessary interaction between asparagine and the hydroxyamino acid or a specific conformation during the transferase reaction is prevented [3].
3. The inhibition of ^{14}C transfer by the non-acceptor peptides points to a partial recognition of other amino acids by the *N*-glycosyltransferase.
4. The hexapeptide Tyr–Asn–Leu–Thr–Ser–Val together with Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose as glycosyl donor constitutes an excellent system for the examination and characterization of asparagine-*N*-glycosyltransferase in rat liver.

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Table 1
Glycosyl acceptor efficiency of synthetic peptide substrates

Amino acid sequence	Conc. (mM)	^{14}C incorporation into			
		Peptide (cpm)	(pmol)	Endogenous protein (cpm)	(pmol)
Control experiment	–	–		2900	(4.5)
Tyr–Asn–Leu–Thr–Ser–Val	1.4	5800	(9.1)	380	(0.6)
Tyr–Asp–Leu–Thr–Ser–Val	2.0	no acceptor		2300	(3.6)
Tyr–Gln–Leu–Thr–Ser–Val	2.0	no acceptor		2400	(3.7)
Tyr–Asn–Leu–Val–Ser–Val	2.0	no acceptor		2300	(3.6)
Tyr–Asn–Pro–Thr–Ser–Val	2.0	no acceptor		2700	(4.2)

Experimental conditions in section 2; Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose, 7.800 cpm (12.2 pmol); 60 min incubation

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