ENZYMATIC N-GLYCOSYLATION OF SYNTHETIC ASN–X–THR CONTAINING PEPTIDES

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1. Introduction

The role of polyprenol sugar derivatives in the biosynthesis of certain eukaryotic glycoproteins has been partly described [1]. Recently lipid pyrophospho-oligosaccharides, postulated as necessary intermediates to initiate N-glycosylation, were shown, when supplied to microsomes, to be effective donors to proteinic acceptors presenting at least one vacant Asn–X–$^{Thr}_{Ser}$ sequence: unfolded forms of proteins [2], polypeptide fragments from CNBr cleavages [3] or a synthetic heptapeptide [4]. It was proposed [5] on the grounds of structural examination of numerous glycoproteins, that this basic tripeptide sequence was a necessary, although probably not sufficient, requirement for an asparagine residue to be N-glycosylated.

The data we report here extend our work [4] showing that thyroid rough microsomes catalyze the transfer of oligosaccharide from [Man-^{14}C]oligosaccharide-lipids to a synthetic human thyroglobulin heptapeptide and further to its dinitrophenylated derivative. Our aim has been to further investigate specificity requirements of the oligosaccharide transferase, using as tools the following series of synthetic peptides:

(I)   H–Ala–Leu–Glu–Asn–Ala–Thr–Arg–NH$_2$
(II)   DNP–Ala–Leu–Glu–Asn–Ala–Thr–Arg–NH$_2$
(III)   H–Asn–Ala–Thr–NH$_2$
(IV)   DNP–Asn–Ala–Thr–NH$_2$
(V) DNP–Ala–Leu–Glu–Asn–Pro–Thr–Arg–NH$_2$
(VI)   H–Tyr–Gln–Ser–Asn–Ser–Thr–Met–NH$_2$

Abbreviations DNP-peptide, dinitrophenylated peptide; DMSO, dimethylsulfoxide

Peptides III and IV are shortened forms of the previously used I and II. Peptide V differs from I by a proline in position X. Peptide VI is a sequence of porcine ribonuclease (residues 73–79, cf.[6]).

2. Materials and methods

2.1. Synthetic peptides and derivatives

They were obtained by the solid phase method and purified as in [4]. Dinitrophenylation was as before. Results of amino acid analysis are given in table 1.

Table 1
Amino acid composition of the peptides

Amino acid	Peptide				
	I	II	III	V	VI
Asp	1.0	0.9	1.0	1.0	1.0
Ser					1.6
Thr	1.0	0.9	0.9	1.0	0.9
Glu	1.1	1.1		1.1	1.0
Pro				0.9	
Ala	2.0	1.1	1.1		
Leu	1.0	0.9		1.0	
Met					0.8
Tyr					0.7
Arg	1.0	1.0		1.0	

Determined after acid hydrolysis in 6 N HCl for 24 h at 110°C

2.2. *Assay for transfer of [*Man-^{14}C*]oligosaccharides to exogenous acceptors*

Preparation of the donor, [*Man*-^{14}C]oligosaccharide-lipids, and control of purity as regards dolichol-P-[^{14}C]*Man* contamination, were as in [4]. Incubation assays (with ~250 μg pig thyroid rough microsomal protein in 75 μl final vol.) were as in [4], except for the following points:

(i) Final concentration of DMSO in incubates was increased from 5% to 10%;

(ii) Each assay contained 3–6 × 10^3 cpm of [*Man*-^{14}C]saccharide-lipids (an aliquot quantity issued from 0.5–1.0 mg particulate protein in our working conditions);

(iii) Reactions were stopped by adding ethanol (125 μl) after 30 min instead of 1 h. Up to this time in the presence of 10% DMSO, [*Man*-^{14}C]oligosaccharide transfers were found to increase nearly linearly with time (fig.1, for peptide II);

(iv) [*Man*-^{14}C]oligosaccharide-peptides were, as in [4], isolated from ethanol supernatants by high-voltage paper electrophoresis in formic acid (100–250 min depending on the composition of the peptides);

(v) Radioactivity transfer to endogenous microsomal protein was evaluated as proposed [7]: the precipitates obtained by ethanol were resuspended in the presence of serum albumin (2 mg) in 10% trichloracetic acid and heated at 100°C for 5 min, a treatment that released [*Man*-^{14}C]saccharides from their lipid derivatives. The pellets

were washed twice with 10% trichloracetic acid before solubilization and counting.

When reduced and alkylated bovine α-lactalbumin was tested as acceptor the entire incubation was stopped and precipitated by 10% trichloracetic acid–5% phosphotungstic acid. In that case, after an acid hydrolysis which released lipid-bound saccharides, the residual material has comprised both exogenous and endogenous proteins.

In order to determine for acceptor peptides app. K_m (μM) and V_{max} ([*Man*-^{14}C]oligosaccharide cpm transferred to peptide) experiments were performed with series of synthetic peptides (at increasing concentrations) and aliquots from a given batch of oligosaccharide-lipids. Four independent batches of donor and 3–5 rough microsomal preparations as enzyme were used for each acceptor peptide.

3. Results and discussion

Table 2 summarizes mean values obtained for app. K_m and V_{max} for synthetic peptides. V_{max}/K_m ratios provide some evaluation of the ability of the enzyme to glycosylate the various synthetic substrates. Figure 2 (A–D) exemplifies representative experiments of [*Man*-^{14}C]oligosaccharide transfers to increasing amounts of exogenous acceptor peptides and also shows the radioactivities incorporated during the same assays into acid stable endogenous acceptors, likely glycoproteins ([8] and section 2). The results obtained with reduced-alkylated α-lactalbumin instead of a synthetic peptide are given in fig.2E.
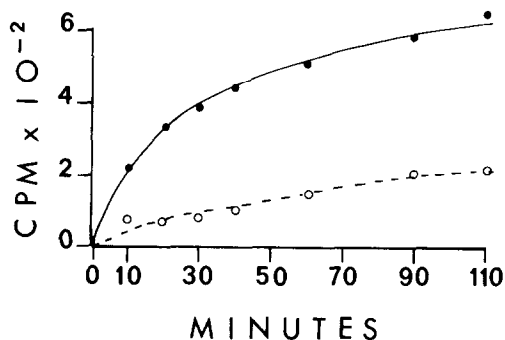

Fig.1. Time course of [*Man*-^{14}C]oligosaccharide transfer to peptide II. Rough microsomal system with 10% DMSO, otherwise as in [4] (530 μM peptide II). Transfer to either exogenous peptide (●——●) or endogenous acceptors (○- - - -○).

Table 2
Compared efficiencies of the synthetic peptides as substrates for glycosylation

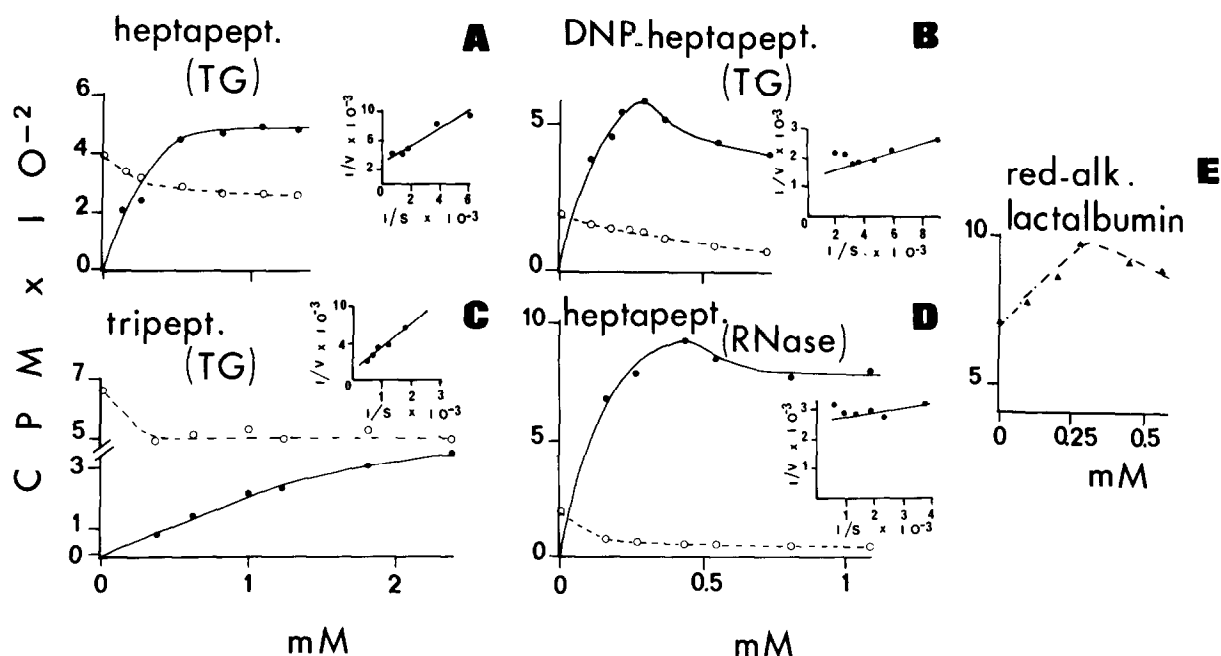| Peptide	app. K_m (μM)	V (cpm)	Ratio V/K_m
I	358 (± 29)	385 (± 38)	1.07
II	72 (± 7)	493 (± 115)	6.84
III	2090 (± 79)	755 (± 382)	0.36
IV	non-acceptor		
V	non-acceptor		
VI	80 (± 5)	900 (± 100)	11.25

Means of 3 independent experiments, and SEM

Fig.2. Effect of increasing the concentration of acceptor substrates on [Man-^{14}C]oligosaccharide transfers in representative experiments. Exogenous peptides were: (●——●) A–D, respectively, peptides I, II, III, VI. Endogenous labels (o- - - -o). The inserts are the Lineweaver-Burk plots for oligosaccharide transfers to peptides. In E (△— · —△) label transferred to reduced alkylated α-lactalbumin and endogenous acceptors taken together. Otherwise as in section 2.

3.1. Efficient acceptors: broad specificity of the transferase

It is interesting that peptide III, the minimal tripeptide sequence that was assayed, was an acceptor for [Man-^{14}C]oligosaccharides although with the poorest affinity and by far the lowest V_{max}/K_m ratio. This suggests some chain length requirement for optimal binding to the transferase. Peptide VI, the ribonuclease heptapeptide was the best substrate and has the highest V_{max}/K_m ratio. Its K_m was close to that of peptide II, the DNP-heptapeptide from thyroglobulin and several-fold less than that of peptide I, the unsubstituted thyroglobulin peptide. We have reproducibly observed an inhibition by excess of substrate for the best substrates (fig.2B,D). In fact the various acceptors listed in table 2 differed more in their K_m than in their V_{max}, except perhaps for peptide VI.

In [4] we have commented upon the increased efficiency conferred on the thyroglobulin heptapeptide by attachment of a lipophilic DNP-group:

it probably results from a facilitated access to the membranous enzyme. In peptide VI the aromatic tyrosine residue might somewhat similarly favour the final binding to the transferase.

It is also worth considering for peptide VI that according to the predictive Chou and Fasmann method its sequence would be highly favourable to the formation of a β-turn, with asparagine at the position $i + 2$ [9,10]. Whether or not a β-turn, i.e., a chain reversal, in such a position contributes per se to recognition by the enzyme cannot be concluded at that time. One might only comment on the fact that it has been found recently that carbohydrate linkages in glycoproteins do seem to occur predominantly around β-turns [9,10] themselves likely situated on the outside of the protein molecule.

As shown in fig.2E, reduced-alkylated α-lactalbumin (2 potential Asn–X–Ser sequences/mol, cf. [11]) also acted as an acceptor when assayed in our system, as it was the case with oviduct particles [2,3]. The order of magnitude of its K_m might approximate

that for peptide I; no calculation was made because we did not know whether 1 or 2 Asn−X−Ser sequences have been glycosylated.

3.2. *Labeling of endogenous acceptors*

An inverse relationship has been observed between transfer to synthetic peptides and labeling of endogenous acceptors: this effect is well illustrated in fig.2B,D for the two best synthetic substrates. It probably results from some competition at the binding site of the enzyme.

3.3. *Non-acceptor peptides*

Peptide IV which differs from I by the presence of a proline between asparagine and threonine was not an acceptor (table 2). This demonstrates that either the conformational constraint introduced by the cyclic side-chain of proline or less probably, the disparition of an amide proton, renders this new peptide unable to interact with the catalytic site. We have nevertheless noticed a slight but reproducible (~25%) inhibition of endogenous protein glycosylation in its presence (data not shown) which suggests that a partial recognition by the enzyme has occurred. It has been a general observation when considering structures of glycoproteins that proline was never found in those Asn−X−$\frac{Thr}{Ser}$ sequences known as glycosylated [5]. In a survey of α-chains of IgA immunoglobulins,one case has been encountered where a mutation from proline to serine has paralleled the apparation of an asparagine-linked sugar moiety [12].

The fact that peptide IV, the DNP-derivative of III was not glycosylated (table 2) certainly results from steric hindrance. Here again an (~50%) inhibition of endogenous protein glycosylation was observed in its presence, thus suggesting partial recognition by the transferase.

4. Conclusions

The results reported here support the following conclusions:
1. A tripeptide sequence such as Asn−Ala−Thr is sufficient for N-glycosylation to occur.
2. The affinity of the oligosaccharide transferase increases with the length of the acceptor peptide

but nevertheless the enzyme is of relatively broad specificity, as concluded [2,3].

Whether or not a conformational bend such as a β-turn near to asparagine favours glycosylation in the case of small substrates (and eventually nascent chains, [13]) remains an open problem.
3. Introducing a proline residue between asparagine and the hydroxy-amino acid renders the tripeptide sequence inadequate for attachment of a saccharide moiety.
4. The porcine ribonuclease synthetic peptide (residues 73−79) constitutes a substrate of choice for routine assays of the transferase.

Acknowledgements

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