The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the *N*-glycosylation step during glycoprotein biosynthesis

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(Received 18 December 1980/Accepted 6 February 1981)

The catalytical role of the hydroxy amino acid in the 'marker sequence' Asn-Xaa-Thr(Ser) for the *N*-glycosylation step of glycoprotein formation was investigated by using a series of hexapeptides derived from Tyr-Asn-Gly-Xaa-Ser-Val by substituting threonine, serine, cysteine, valine and *O*-methylthreonine respectively for Xaa. The results, which were obtained with calf liver microsomal fractions as enzyme source and dolichyl diphosphate di-*N*-acetyl[¹⁴C]chitobiose as glycosyl donor showed that the threonine-, serine- and cysteine-containing derivatives could be glycosylated, although at very different rates, whereas the valine and *O*-methylthreonine analogues did not work as glycosyl acceptors. Replacement of threonine by serine resulted in a 4-fold decrease in $V_{\max}$ and about a 10-fold increase in K_m for glycosyl transfer. Replacement of serine by cysteine again decreased acceptor activity 2–3-fold. The various results, taken together, indicate an absolute requirement for a hydrogen-bond-donor function in the side chain of the hydroxy amino acid of the 'marker sequence' and furthermore, point to a considerable influence of the structure of this amino acid on binding as well as on the glycosyl transfer itself. In order to explain the observed differences in the glycosyl-transfer rates, a model is proposed with a hydrogen-bond interaction between the amide of asparagine as the hydrogen-bond donor and the oxygen of the hydroxy group of the hydroxy amino acid as the hydrogen-bond acceptor. The participation of the hydroxy group in the catalytic mechanism of glycosyl transfer in a kind of a proton-relay system is discussed.

The biosynthesis of asparagine-linked saccharide chains of glycoproteins is thought to occur in the endoplasmic reticulum and Golgi apparatus via a reaction sequence involving dolichyl mono- and di-phosphate sugar derivatives (for review, see Elbein, 1979; Parodi & Leloir, 1979). Although detailed information is available on the enzymes concerned with the synthesis of these glycolipid intermediates, our knowledge about the specificity of enzymes transferring the lipid-bound oligo-saccharide to protein and on the nature of the polypeptide acceptor itself is rather limited.

Some years ago, it was noted that asparagine residues bearing carbohydrate chains are invariably followed by a hydroxy amino acid in the position

next but one on the C-terminal side (Neuberger & Marshall, 1968). On the basis of this observation it was postulated that the triplet sequence Asn-Xaa-Thr(Ser) is a necessary, though not in itself sufficient, prerequisite for *N*-glycosylation to occur (Marshall, 1972, 1974). Indeed, recent work in our laboratory supported this idea of a 'marker sequence' when we investigated structural requirements for *N*-glycosylation with exogenous synthetic peptides of known amino acid composition and defined sequence as glycosyl acceptors (Bause, 1979). By using these peptide substrates as probes, we were able, in addition, to demonstrate that the position Xaa in the 'marker sequence' could be occupied by any amino acid except proline. Furthermore, our studies pointed to a minimum chain length of a pentapeptide for glycosylation to occur, indicating an absolute requirement for at least one amino acid on either side of the triplet sequence for binding of

Abbreviations used: Dol-*P*, dolichyl monophosphate; Dol-*PP*, dolichyl diphosphate; (GlcNAc)₂, di-*N*-acetylchitobiose; Xaa, 'unknown' or 'variable' amino acid residue.

the substrate to the *N*-glycosyltransferases (Bause & Hettkamp, 1979).

The present paper is concerned with a more detailed analysis of the necessity and role of the hydroxy amino acid in the Asn-Xaa-Thr(Ser) sequence for the catalytic mechanism of *N*-glycosyltransferases. We have studied this question by using a series of hexapeptide derivatives that differ from each other by an exchange in the hydroxy amino acid position. The results are interpreted in terms of a 'marker-sequence' requirement and its consequence for the catalytic action of the enzymes under investigation.

Materials and methods

Materials

UDP-*N*-acetyl[^{14}C]glucosamine (323 Ci/mol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Dol-*P*, UDP-*N*-acetylglucosamine, *N*-*t*-butoxycarbonyloxyimino-2-phenylacetoneitrile and *O*-methylthreonine were from Sigma. Bio-Beads S-X1 (chloromethylated) were purchased from Bio-Rad and *t*-butoxycarbonyl amino acids from Serva. All other chemicals were purchased from commercial sources and were of the highest purity available.

Methods

Preparation of calf liver microsomal membranes. These were prepared as described by Moulé *et al.* (1960). The final preparation contained approx. 20 mg of protein/ml when assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

***N*-Glycosyltransferase assay.** Glycosylation of peptides was measured with Dol-*PP*-[^{14}C](GlcNAc)₂ as donor, essentially as described by Bause & Hettkamp (1979). When the cysteine-containing peptide was tested for glycosyl-acceptor activity, the standard incubation medium was made 2 mM with respect to dithiothreitol to prevent oxidation of the thiol function. The addition of dithiothreitol was shown to have no effect on *N*-glycosyltransferase activity when measured with Tyr-Asn-Gly-Thr-Ser-Val as acceptor peptide. The reaction products were isolated either by electrophoresis at pH 1.9 in formic acid on cellulose plates or by partition (Bause & Lehle, 1979).

Synthesis and purification of peptides. Peptides were synthesized by the solid-phase method described by Merrifield (1963) and Erickson & Merrifield (1976). Cysteine-containing peptides were cleaved from the resin with anhydrous HF at 0°C for 90 min in the presence of anisole (methoxybenzene). Purification was achieved by gel-permeation chromatography on Bio-Gel P4 in 1 M-acetic acid as solvent. The *O*-methylthreonine-containing

peptide was further purified by preparative chromatography on silica-gel layers in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase).

***t*-Butoxycarbonylation of *O*-methylthreonine.** *t*-Butoxycarbonylation was performed as follows. A mixture of 2 mmol of *O*-methylthreonine, 3 mmol of triethylamine and 2.2 mmol of *N*-*t*-butoxycarbonyloxyimino-2-phenylacetoneitrile in 5 ml of aq. 50% acetone was made to react for 4 h at room temperature. After the solvent had been evaporated under reduced pressure, the remaining residue was resuspended in 10 ml of water and the aqueous phase extracted four times with methyl acetate after the pH of the solution had been adjusted to about 1.8 with HCl. The combined organic phases were dried with Na₂SO₄, filtered and evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of methyl acetate and crystallized by the addition of a mixture of ether/light petroleum (b.p. 60–90°C). Recrystallization yielded 340 mg of chromatographically pure *t*-butoxycarbonyl-*O*-methylthreonine, N.m.r. data are in agreement with the expected structure. The m.p. was 130°C (uncorrected).

Other methods. Preparation and purification of Dol-*PP*-[^{14}C](GlcNAc)₂ was as described by Bause & Hettkamp (1979). Radioactivity was detected either by radioautography with Sakura QH X-ray film or measured in a liquid-scintillation counter (Delta 300; Searle Analytic Inc.). Amino acids were analysed on a Beckmann amino acid analyser, model 119 CL, after hydrolysis of peptides with 5.7 M-HCl for 20 h.

Results and discussion

Characterization of glycosyl transfer to Tyr-Asn-Gly-Thr-Ser-Val, Tyr-Asn-Gly-Ser-Ser-Val and Tyr-Asn-Gly-Cys-Ser-Val

In order to investigate the role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the catalytic action of *N*-glycosyltransferases, we have synthesized two families of hexapeptides derived from Tyr-Asn-Gly-Xaa-Ser-Val. The first one contains either threonine, serine or cysteine in position Xaa, the second one, valine or *O*-methylthreonine. Both groups differ by their capability of participating in hydrogen-bond interactions. Although the hydroxy groups of threonine and serine and, though to a lesser extent, the thiol group of cysteine are able to function as hydrogen-bond acceptors as well as hydrogen-bond donors, the *O*-methylthreonine derivative is a hydrogen-bond acceptor only and the side chain of valine may not be hydrogen-bonded at all.

When calf liver microsomal fractions are incubated with Tyr-Asn-Gly-Thr-Ser-Val in the presence

Fig. 1. Time course of glycosyl transfer from Dol-PP-di-N-acetyl[^{14}C]chitobiose to exogenous peptides catalysed by calf liver membranes

Incubations were performed with 2000 c.p.m. of Dol-PP-[^{14}C](GlcNAc) $_2$ in the presence of 50 mM-Tris/HCl, pH 7.5, 0.8% Triton X-100, 10 mM-MnCl $_2$, 0.2 mg of membrane protein and 2 mM-peptide in a total volume of 50 μl (●, Tyr-Asn-Gly-Thr-Ser-Val; ○, Tyr-Asn-Gly-Ser-Ser-Val; △, Tyr-Asn-Gly-Cys-Ser-Val). Glycopeptide formation was assayed by partition as described by Bause & Lehle (1979).

Fig. 2. Glycosylation of Tyr-Asn-Gly-Thr-Ser-Val as a function of peptide concentration

Reaction mixtures containing 50 mM-Tris/HCl, pH 7.5, 0.8% Triton X-100, 10 mM-MnCl $_2$, 600 c.p.m. of Dol-PP-[^{14}C](GlcNAc) $_2$, 0.2 mg of membranes and peptide as indicated were incubated for 10 min at room temperature. Glycosyl transfer to peptide was measured by the partition procedure as described by Bause & Lehle (1979).

of Dol-PP-[^{14}C](GlcNAc) $_2$ as glycosyl donor, a rapid transfer of [^{14}C](GlcNAc) $_2$ to peptide is found (Fig. 1). The deviation of glycosyl transfer rate from linearity, which is observed after an incubation time of about 10 min, is probably the result of substrate depletion. The glycosyltransferase activity is stimulated by bivalent metal ions, 10 mM-Mn $^{2+}$ being most effective (results not shown). When glycosyl transfer is measured as a function of peptide concentration, saturation kinetics is observed (Fig. 2). Evaluation by the Lineweaver-Burk procedure reveals a K_m value of 0.16 mM and a $V_{\max}$ of 2800 c.p.m./10 min (see the insert to Fig. 2). These values are close to those found in rat liver for Tyr-Asn-Leu-Thr-Ser-Val, indicating a similar specificity of *N*-glycosyltransferases in rat and calf liver (Bause, 1979).

The occurrence of glycoproteins with glycosylated Asn-Xaa-Ser sequences suggested that a glycosylation of Tyr-Asn-Gly-Ser-Ser-Val should also be possible *in vitro*. Indeed, we found a time-dependent glycosyl transfer when the hexapeptide was incubated with calf liver microsomal fraction and Dol-PP-(GlcNAc) $_2$ as donor (Fig. 1). In comparison with the result for the threonine analogue, however, the apparent K_m value was about 10 times higher, whereas $V_{\max}$ was decreased 4-fold (see Table 1). This result clearly points to a substantial influence of the methyl group on both binding of the peptide substrate and catalysis. With respect to available sequence data, it is striking that the ratio of glycosylated Asn-Xaa-Thr and Asn-Xaa-Ser sequences *in vivo*, is lower than expected from the glycosylation rates *in vitro* of corresponding model peptides (Hunt & Dayhoff, 1970; Beeley, 1977).

In contrast to the observation of a number of glycosylated Asn-Xaa-hydroxy amino acid sequences, no natural Asn-Xaa-Xaa-Cys structure is known to date with an asparagine residue bearing a carbohydrate chain. However, our studies revealed that, at least under conditions *in vitro*, the Tyr-Asn-Gly-Cys-Ser-Val peptide functions as *N*-glycosyl acceptor. The transfer of di-N-acetyl[^{14}C]chitobiose from the glycolipid to this peptide was found to be relatively low, but reproducible (Fig. 1). This surprising result clearly indicates that, in addition to an Asn-Xaa-hydroxy amino acid 'marker sequence', the Asn-Xaa-Cys triplet is also capable of fulfilling conformational and catalytic prerequisites to be recognized and glycosylated by corresponding *N*-glycosyltransferases, suggesting that, *in vivo*, a glycosylation should, in principle, be possible. This assumption is supported by a comparison of the standardized transfer rates of the Asn-Gly-Cys and Asn-Gly-Ser hexapeptides, which differ by a factor of 2–3 only (Table 1). An attempt to explain the apparent lack of glycosylated Asn-Xaa-Cys sequences *in vivo* must take the following into

Table 1. *Glycosyl-acceptor capabilities of various hexapeptides with a modified 'marker sequence'*

Relative *N*-glycosyltransferase activities ($V_{\max}/K_m$) are based on the activity with the serine-containing peptide. Abbreviation used: n.d., not determined.

Amino acid sequence	K_m (mM)	$V_{\max}$ (c.p.m./10 min)	Relative transferase activity
Tyr-Asn-Gly-Thr-Ser-Val	0.16	2080	40
Tyr-Asn-Gly-Ser-Ser-Val	1.6	550	1
Tyr-Asn-Gly-Cys-Ser-Val	n.d.	n.d.	0.4
Tyr-Asn-Gly-Val-Ser-Val	—	—	0
Tyr-Asn-Gly-Thr-Ser-Val OCH ₃	—	—	0

consideration. First, glycosylated asparagine residues in such a triplet have not been supposed likely to exist until now. It may be necessary, therefore, to recheck the sequence data for the glycopeptides. Secondly, the statistical occurrence of cysteine, and consequently its frequency in Asn-Xaa-Cys structures, are considerably lower in comparison with Asn-Xaa-Thr and Asn-Xaa-Ser sequences respectively. Thirdly, cysteine and its disulphide derivative accomplish another, more specifically structural, function in the protein molecule.

Non-acceptor peptides

The results summarized in Table 1 demonstrate that an exchange of the hydroxy function of threonine in the 'marker sequence' by a methyl group (threonine versus valine) or methylation of the hydroxy group to give *O*-methylthreonine abolishes acceptor capabilities of corresponding peptides completely. These findings prove the substantial requirement for a hydrogen-donor function in the side chain of this amino acid. Indeed, this prerequisite is fulfilled by threonine, serine and cysteine.

pH-dependence of *N*-glycosyltransferase

N-Glycosylation of the amide function of asparagine requires a nucleophilic attack of the nitrogen electron pair on the phosphate-ester-activated C-1 of the glycosyl donor. As a consequence, transglycosylation must be accompanied by a proton transfer to a basic group that should manifest itself if it ionizes in the pH range accessible to investigation. By using Tyr-Asn-Gly-Thr-Ser-Val as acceptor peptide, a sharp sigmoidal increase of glycosyl transfer activity is observed with increasing pH (Fig. 3). The most simple explanation for this observation is the participation of the side chain of a histidine residue as the proton acceptor during catalysis. A similar pH curve is reported for an oligosaccharide transferase from thyroid (Ronin, 1980).

Fig. 3. *pH-dependence of N-glycosyltransferase activity* Membranes were incubated with 2000 c.p.m. of Dol-PP-[¹⁴C](GlcNAc)₂ in the absence (O) and presence (●) of 1 mM-Tyr-Asn-Gly-Thr-Ser-Val. Incubations were terminated after 20 min by the addition of 1 ml of methanol, and glycosyl transfer was assayed by partition (Bause & Lehle, 1979).

General conclusions

Studies *in vitro* on the substrate specificity of *N*-glycosyltransferases in rat liver had demonstrated that asparagine residues with a proline residue as the direct *C*-terminal neighbour (Xaa) could not be glycosylated, despite the presence of an Asn-Xaa-Thr sequence as necessary prerequisite for *N*-glycosylation (Bause, 1979). Our interpretation of this finding was that an interaction between the side chains of asparagine and the hydroxy amino acid, apparently necessary for binding of the peptide substrate and/or catalysis, is prevented by the particular structure of the imino acid. The data, which are summarized in Table 1, indicate that this essential interaction might be due to the capability of the hydroxy amino acid in the above 'marker sequence' to function as hydrogen-bond donor. This

can be concluded from the observation that only peptide derivatives with this structural feature act as glycosyl acceptors (i.e. Tyr-Asn-Gly-Thr-Ser-Val and the serine- and cysteine-containing analogues respectively). The apparent significance of a hydrogen-bond-donor function for the enzymic glycosyl transfer makes the observed glycosylation of the peptide containing the 'marker sequence' Asn-Gly-Cys more understandable and, in addition, less surprising. Up to now, however, no glycoprotein

with a glycosylated triplet sequence of this type has been detected in Nature.

The marked differences in the glycosylation rates of the various acceptor peptides furthermore point to a complex influence of the nature of the side chain of the amino acid in the hydroxy amino acid position of the 'marker sequence' on binding as well as on the glycosylation step, i.e. on the rate of sugar transfer itself. Thus the lack of the methyl group (substitution of threonine by serine) leads to a striking increase in K_m and decrease in V_{max} , resulting in a factor of 40 when enzyme effectivity is given by V_{max}/K_m , whereas replacing serine with cysteine decreases the initial glycosylation rate only 2–3-fold.

Attempts to interpret the acid–base-titration behaviour of egg-albumin glycopeptides led to the construction of a model in which the hydroxy group of the hydroxy amino acid of the 'marker sequence' Asn-Xaa-Thr(Ser) was hydrogen-bonded to the amide oxygen of the asparagine residue that was glycosylated (Marshall, 1972). It was speculated that this type of hydrogen-bond interaction should also be applicable to an apoprotein, thus helping to increase the acid dissociation constant of the amide group in the asparagine side chain, thereby facilitating the substitution of one of the hydrogen atoms by a glycosyl residue (Marshall, 1974; Fig. 4, structure A). Although this model can be used to explain the function of the 'marker sequence' as 'recognition site', it does not describe correctly the catalytic participation of the hydroxy amino acid in the mechanism of glycosyl transfer. For chemical reasons one must assume that the amide function of asparagine may not undergo glycosylation without a preceding activation. In the Marshall (1972) model (Fig. 4, structure A), the orientation of the hydrogen bond gives rise to a decrease in nucleophilicity of the nitrogen electron pair with a corresponding increase in N–H acidity. Under these conditions a nucleophilic attack on the Dol-PP-bound sugar derivative can only be expected when it is preceded by a proton dissociation from the amide group. However, this deprotonation as the first step of glycosyl transfer seems to be highly unlikely, because of the relatively low base strength of potential proton acceptors, represented by side chains of the amino acids (i.e. carboxylate, thiolate or imidazole residues). The results of our studies are in fact at variance with a reaction sequence that follows from the Marshall (1972) model. So we found that, in comparison with the threonine and serine derivatives, the cysteine-containing hexapeptide, despite the relatively high acidity of a thiol group, which might favour the above-discussed mechanism, was glycosylated only to a small extent. Similarly, glycosyl transfer to the threonine-containing peptide was faster than to the serine-containing one, even though the +I-effect [positive inductive (electron-donating)

Fig. 4. Models representing possible hydrogen-bonded interactions between the side chains of asparagine and the hydroxy amino acid in the 'marker sequence' Asn-Xaa-Thr(Ser)

Structure A (proposed by Marshall, 1972) incorporates a hydrogen bond between the hydroxy function of the hydroxy amino acid and the carbonyl-group oxygen atom of the asparagine residue. In structure B, the amide group of asparagine functions as hydrogen-bond donor and the hydroxy-group oxygen atom of the hydroxy amino acid as hydrogen-bond acceptor. The significance of both types of interactions for the catalytic mechanism of enzymic glycosyl transfer is discussed in the text.

effect] of the methyl group of threonine should result in a decreased N-H acidity and consequently lower sugar-transfer rate.

The obvious contradictions between our results and a mechanism deduced from the Marshall (1972) model initiated the construction of an alternative model, which is outlined in Fig. 4, structure B. Here, the hydroxy amino acid side chain is acting as hydrogen-bond acceptor, whereas the amide group of asparagine functions as hydrogen-bond donor. As a result of this interaction the nucleophilicity of the amide electron pair is increased, thus giving rise to a higher reactivity towards the glycosyl donor. On the other hand, proton transfer in the transition state to a basic group of the enzyme in a kind of proton-relay system would be facilitated. For this proton-relay system, the observed differences in the glycosylation rates are compatible with the expected decrease in base strength of the hydrogen-bond-acceptor functions (cysteine < serine < threonine). The particular efficiency of the threonine-containing peptide to act as glycosyl acceptor could be explained by the fact that the +I-effect of the methyl group might increase the proton-acceptor capability of the hydroxy group. Finally, the lower ability of sulphur to function as hydrogen-bond acceptor would consequently result in a lower glycosylation rate, which is observed.

E. B. thanks Professor F. W. Hemming and Dr. A. Warren (University of Nottingham) for fruitful dis-

cussions. The work was supported by a grant from the 'Deutsche Forschungsgemeinschaft'.

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