Investigation of the active site of oligosaccharyltransferase from pig liver using synthetic tripeptides as tools

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Oligosaccharyltransferase (OST), an integral component of the endoplasmic-reticulum membrane, catalyses the transfer of dolichyl diphosphate-linked oligosaccharides to specific asparagine residues forming part of the Asn-Xaa-Thr/Ser sequence. We have studied the binding and catalytic properties of the enzyme from pig liver using peptide analogues derived from the acceptor peptide *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ by replacing either asparagine or threonine with amino acids differing in size, stereochemistry, polarity and ionic properties. Acceptor studies showed that analogues of asparagine and threonine with bulkier side chains impaired recognition by OST. Reduction of the β-amide carbonyl group of asparagine yielded a derivative that, although not glycosylated, was strongly inhibitory (50% inhibition at ≈ 140 μM). This inhibition may be due to ion-pair formation involving the NH_3^+ group and a negatively charged base at the active site. Hydroxylation of asparagine at the β-C position increased K_m and decreased $V_{max.}$, indicating an effect on both binding and catalysis. The *threo* configuration at the β-C atom of the hydroxyamino acid was essential for substrate binding. A peptide derivative obtained by replacement of the threonine β-hydroxy group with an NH_2 group was found to display acceptor activity. This shows that the primary amine is able to mimic the hydroxy group during transglycosylation. The pH optimum with this derivative is shifted by approximately 1 pH unit towards the basic region, indicating that the neutral NH_2 group is the reactive species. The various data are discussed in terms of the catalytic mechanism of OST, particular emphasis being placed on the role of threonine/serine in increasing the nucleophilicity of the β-amide of asparagine through hydrogen-binding.

INTRODUCTION

Oligosaccharyltransferase (OST), a key enzyme in the pathway of *N*-glycoprotein biosynthesis, catalyses the formation of N-glycosidic linkages between carbohydrate and polypeptide chains. The glycosyl donor in this reaction is a dolichyl diphosphate (Dol-PP)-linked $GlcNAc_2$-Man_9-Glc_3 oligosaccharide which is transferred 'en bloc' to the β-amide function of specific asparagine residues. The protein-bound precursor oligosaccharide is subsequently processed by the concerted action of several glycosidases and glycosyltransferases, finally yielding the mature glycan structure [1].

It is now well established that a signal sequence of the type Asn-Xaa-Thr/Ser is necessary, although in itself not sufficient, as a prerequisite for N-glycosylation [2–4]. Model studies on the functional role of this triplet sequence support the view that the hydoxyamino acid not only serves as a recognition signal but participates actively in transglycosylation, probably by enhancing the nucleophilicity of the β-nitrogen of the acceptor asparagine through hydrogen-bond formation [5,6]. It is apparent that this interaction requires a specific conformation of the polypeptide chain at the glycosylation site, and evidence is accumulating that β-turns, loop structures and Asx turns may be potential candidates [7–10].

Because of its unique specificity, the activity of OST can be determined reliably in crude microsomal fractions using Dol-PP-oligosaccharides as glycosyl donors and synthetic peptides, containing the Asn-Xaa-Thr/Ser motif, as acceptors. Studies with peptide substrates differing in chain length and amino acid sequence have shown that their acceptor properties are not severely affected by the nature of the Xaa amino acid or of those framing the 'marker sequence' [11,12]. An exception is when there is a proline residue in the Xaa position or on the C-terminal side of the hydroxyamino acid [2,8,13]. In these cases the peptide is neither glycosylated nor bound by the enzyme, suggesting that proline may trigger the conformation of the peptide backbone in a manner that prevents the postulated interaction between the acceptor asparagine and the hydroxyamino acid. The observation that Asn-Pro-Thr/Ser and Asn-Xaa-Thr/Ser-Pro sites have never been found to be glycosylated supports this view, further indicating that data from *in vitro* model studies can be transferred to the situation *in vivo* [13].

In order to verify the functional role of the hydroxyamino acid during catalysis and to gain more insight into the nature of the active site of OST, we synthesized two series of tripeptides containing amino acids structurally modified in either the asparagine or threonine position of the Asn-Xaa-Thr sequence. We then analysed the effect of these modifications on binding and glycosylation properties, allowing a number of conclusions to be drawn with respect to the mechanism of OST as well as to the active-site architecture of the enzyme.

MATERIALS AND METHODS

Materials

Materials and chemicals were obtained from the following sources: UDP-*N*-acetyl[^{14}C]glucosamine (specific radioactivity 323 Ci/mol), Amersham; Dol-P, Triton X-100, UDP-*N*-acetylglucosamine, Sigma; L-amino acids, L-allothreonine, L-homoserine, L-phenylserine, trifluoroacetic acid, di-*t*-butylpyrocarbonate [$(Boc)_2O$], 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline, *NN'*-dicyclohexylcarbodiimide, Fluka; benzyloxycarbonyl hydrazide (Z-$NHNH_2$), *N*-α-Boc-N-γ-*Z*-αγ-L-diaminobutyric acid (DABA), L-aspartic acid β-methyl ester, Bachem; silica-gel G 60 plates, Merck. L-Cysteinesulphonamide was kindly provided by Dr.

Abbreviations used: OST, oligosaccharyltransferase; Dol-PP, dolichyl diphosphate; DABA, diaminobutyric acid.

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Assay of OST and inhibition studies

Standard incubation mixtures for measuring peptide glycosylation contained in a total volume of 100 μl: 50 mM Tris/HCl, pH 7.2, 10 mM $MnCl_2$, 0.8% Triton X-100, 150 mM sodium acetate, 1 M sucrose, 0.3% phosphatidylcholine, 3000 c.p.m. of Dol-PP-[^{14}C]$GlcNAc_2$ and peptide. For those peptides with poor solubility, we used DMSO/water (1:1, v/v) for stock solutions, giving 10% (v/v) DMSO in the final incubation mixture. The reactions, started by the addition of pig liver OST purified as in [14], were conducted at 25 °C and stopped by the addition of 0.5 ml of methanol. After centrifugation the supernatant was removed and made biphasic by the addition of 0.75 ml of chloroform and 0.15 ml of water. [^{14}C]Glycopeptides present in the aqueous upper phase were then determined by liquid scintillation counting. Unless stated otherwise, the incubation time was 30 min and the concentration of peptide derivatives in the assay 10 mM. Binding/inhibitory properties were determined by incubating peptide I (1.0 mM) under standard assay conditions for 10 min in the presence of the respective non-acceptor peptides (10 mM) (or various concentrations of the inhibitor VIII); the reaction mixtures were processed as described above. $V_{max.}$ values, given as c.p.m./min, represent relative values, determined under identical reaction conditions and using the same enzyme preparation.

Peptide synthesis

All tripeptides, except derivatives VII and XIV, were synthesized in solution, using adaptations of published procedure [15,16]. The α-amino group was protected by di-*t*-butyloxycarbonylation (α-N-Boc) and side-chain NH_2 functions by benzyloxycarbonylation (N-β/γ-Z). Deblocking was carried out with trifluoroacetic acid (Boc derivatives) or by hydrogenation (Z derivatives). Peptide bonds were synthesized either using 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline in ethanol as coupling reagent or by the active ester method using *NN'*-dicyclohexylcarbodi-imide and auxiliary nucleophiles for activation. The purity of the peptides was checked by TLC on silica gel G 60 using two solvent systems [butan-1-ol/acetic acid/water (4:1:1, by vol.) and chloroform/methanol/acetic acid (65:25:5, by vol.)], and their structures were confirmed by ^{1}H-NMR and MS. *N*-Benzoyl-Asn-Gly-Thr-$NHCH_3$ (I): MH^+ *m/z* 408; ^{1}H-NMR δ 1.0 (β-CH_3), 2.56 (amide-CH_3), 2.65 (β-CH_2-Asn), 3.78 (CH_2-Gly), 4.04 (β-CH-Thr), 4.07 (α-CH-Thr), 4.73 (α-CH-Asn), 7.47–7.89 (C_6H_5). Selected ^{1}H-NMR data for amino acid analogues and molecular masses of tripeptide derivatives were as follows: Peptide II: MH^+ *m/z* 424. Peptide III: MH^+ *m/z* 424. Peptide IV: MH^+ *m/z* 444; ^{1}H-NMR δ 3.65 (β-CH_2-$CysSO_2NH_2$), 4.82 (α-CH-$CysSO_2NH_2$). Peptide V: MH^+ *m/z* 409. Peptide VI: MH^+ *m/z* 423. Peptide VII: MH^+ *m/z* 424. Peptide VIII: MH^+ *m/z* 394; ^{1}H-NMR δ 2.1 (β-CH_2-DABA), 2.95 (γ-CH_2-DABA), 4.55 (α-CH-DABA). Peptide IX: MH^+ *m/z* 394; ^{1}H-NMR δ 1.95 (β-CH_2-homo-Ser), 3.5 (γ-CH_2-homo-Ser), 4.5 (α-CH-homo-Ser). Peptide X: MH^+ *m/z* 394; ^{1}H-NMR δ 3.6 (β-CH_2-Ser), 4.2 (α-CH-Ser). Peptide XI: MH^+ *m/z* 408; ^{1}H-NMR: δ 1.0 (β-CH_3-allo-Thr, 3.87 (β-CH-allo-Thr), 4.13 (α-CH-allo-Thr). Peptide XII: MH^+ *m/z* 437. Peptide XIII: MH^+ *m/z* 470; ^{1}H-NMR δ 4.30 (β-CH-phenyl-Ser), 4.72 (α-CH-phenyl-Ser), 7.18–7.35 (C_6H_5-phenyl-Ser). Peptide XIV: MH^+ *m/z* 406; ^{1}H-NMR δ 2.1 (β-CH_3-keto derivative), 4.98 (α-CH-keto derivative). Peptide XV: MH^+ *m/z* 407; ^{1}H-NMR δ 0.95 (β-CH_3-DABA), 3.18 (β-CH-DABA), 4.08 (α-CH-DABA). Peptide XVI: MH^+ *m/z* 407; ^{1}H-NMR δ 0.98 (β-CH_3-DABA), 3.32 (β-CH-DABA), 4.32 (α-CH-DABA).

Synthesis of amino acid analogues

Racemic *threo/erythro*-β-hydroxyasparagine

D/L-*Threo*- and *erythro*-β-hydroxyaspartic acid were prepared from *cis*- and *trans*-epoxysuccinic acid respectively by treatment with concentrated NH_4OH as described previously [17]. The β-carboxy function was esterified with benzyl alcohol and the isolated β-benzyl ester, after crystallization from water, converted into the β-acid amide by treatment with 25% NH_4OH as detailed in [18]. Crystallization from hot water yielded a racemate of D/L-*threo*- and *erythro*-β-hydroxyasparagine respectively. *N*-α-Benzoyl-*threo* derivative: ^{1}H-NMR δ 4.45 (β-CH), 4.92 (α-CH), 7.4–8.0 (C_6H_5). *N*-α-Benzoyl-*erythro* derivative: ^{1}H-NMR δ 4.25 (β-CH), 4.95 (α-CH), 7.4–7.9 (C_6H_5).

Racemic *threo/erythro*-$\alpha\beta$-DABA

N-α-Boc-L-threonine methylamide, synthesized as in [15], was oxidized to the β-keto derivative by treatment with acetic acid anhydride in DMSO [19], followed by reduction of the keto function with $NaCNBH_3$ in the presence of ammonium acetate [20]. Although L-threonine was used as starting material, a racemic mixture of *threo/erythro* forms was obtained (i) because of keto–enol tautomerism occurring after α-*N*-Boc-L-threonine-*N*-methylamide oxidation and (ii) because reductive amination is non-stereospecific. After protection of the β-amino function by benzyloxycarbonylation, *threo*- and *erythro*-isomers were isolated from the reaction mixture by fractional crystallization using methanol/ether and further purified by silica-gel chromatography [ethanol/acetonitrile/water (5:5:1, by vol.)]. *N*-α-Boc-β-*Z-threo* derivative: ^{1}H-NMR δ 1.0 (β-CH_3), 1.36 (Boc), 2.52 (amide-CH_3), 3.95 (β-*CH*), 4.0 (α-CH), 4.99 (CH_2-aryl), 6.65–7.35 (C_6H_5). *N*-α-Boc-β-*Z-erythro* derivative: ^{1}H-NMR δ 0.95 (β-CH_3), 1.36 (Boc), 2.53 (amide-CH_3), 3.84 (β-CH), 4.04 (α-CH), 4.98 (CH_2-aryl), 6.68–7.32 (C_6H_5). The α-*N*-Boc group was cleaved with trifluoroacetic acid and the unblocked derivatives were used for peptide synthesis.

Synthesis of the peptide derivatives VII and XIV

The Asp-β-hydroxyamic acid derivative VII (MH^+ 424) was obtained from peptide VI (MH^+ 423) by treatment with NH_2OH. Derivative XIV was synthesized by oxidation with acetic acid anhydride in DMSO of the allothreonine tripeptide XI [19] and purified by silica-gel chromatography using butan-1-ol/acetic acid/water (4:1:1, by vol.) as the solvent. Reduction of the keto function in derivative XIV with $NaBH_4$ yielded a peptide mixture with *N*-glycosyl acceptor properties.

General methods

OST was partially purified from pig liver crude microsomes as described by Breuer and Bause [14]. Dol-PP-[^{14}C]GlcNAc and Dol-PP-[^{14}C]$GlcNAc_2$ were prepared as detailed in [12]. The specific radioactivity of the two [^{14}C]glycolipids is likely to be similar because elongation of preformed Dol-PP-[^{14}C]GlcNAc to yield the [^{14}C]chitobiosyl lipid was achieved by pulse–chasing the incubation with a large excess of unlabelled UDP-GlcNAc. Radioactivity was determined by liquid-scintillation counting using Bray's solution as counting fluid [21]. The molecular mass of peptides (MH^+) was determined on a VG ZAB FAB mass spectrometer; ^{1}H-NMR data were measured in [^{2}H]DMSO/^2H_2O using a Bruker AMX-500 MHz spectrometer.

RESULTS AND DISCUSSION

Design and acceptor properties of *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ (I)

Synthetic peptides, containing the Asn-Xaa-Thr/Ser motif, have been used successfully to characterize the specificity of OSTs of different origin [3,11,12,14,22,23]. Amongst other things, these studies revealed that the shortest peptide unit accepted by OST as a substrate is an Asn-Xaa-Thr/Ser tripeptide, provided that both its N- and C-terminus are blocked by amide formation and that Xaa is not proline [2,3,8,14]. Taking advantage of this observation we synthesized the *N*-benzoylated Asn-Gly-Thr-$NHCH_3$ tripeptide derivative (I) and used this as the standard substrate (Figure 1). The *N*-benzoyl group was introduced because (i) the acceptor properties of peptide I turned out to be severalfold better than those of N-acetylated derivatives, and (ii) the concentration of N-benzoylated intermediates and peptides in solution could be determined conveniently by UV absorption.

Incubation under standard assay conditions of OST, purified from pig liver microsomes [14], with peptide I and Dol-PP-[^{14}C]$GlcNAc_2$ resulted in a rapid and time-dependent formation of ^{14}C-labelled glycopeptide (Figure 2). Treatment of the [^{14}C]glycopeptide with glycopeptidase F released [^{14}C]chitobiose as the only radioactive product, whereas no cleavage occurred by β-elimination, indicating that the [^{14}C]chitobiosyl moiety is N-glycosidically linked to the β-amide nitrogen of asparagine (not shown). Transfer of [^{14}C]chitobiose to peptide I was found to be concentration-dependent, with an apparent peptide K_m of $\approx$ 220 μM and a $V_{max.}$ of $\approx$ 160 c.p.m./min (Figure 3). The K_m of the peptide was not altered when different concentrations of the Dol-PP-[^{14}C]$GlcNAc_2$ donor were used. This suggests that the glycolipid donor and the acceptor peptide form a ternary complex with the enzyme, obviously differing from that proposed for a Ping Pong-type mechanism of transglycosylation [14]. The K_m value of peptide I was of the same order of magnitude as those previously determined for other peptides, including the hexapeptides Ala-Asn-Gly-Thr-Ala-Val, Pro-Asn-Gly-Thr-Ala-Val and Tyr-Asn-Lys-Thr-Ala-Val [8,14]. This indicates that, *in vitro* at least, the acceptor properties are determined mainly by the Asn-Xaa-Thr motif and that amino acids adjacent to the signal sequence are of less importance.

In contrast with Dol-PP-[^{14}C]chitobiose, Dol-PP-[^{14}C]-acetylglucosamine was found to be an extremely poor donor in the OST reaction (Figure 2). Assuming a similar specific radioactivity for the two [^{14}C]glycolipids in the assay (see the Materials and methods section), it can be estimated from the initial glycosylation rates that their donor properties differ by more than 170-fold. Since the sugar moiety in both glycolipids is activated via the same phosphoacetal linkage, it is likely that a specific binding site for the peripheral GlcNAc residue, which is part of the chitobiosyl unit, exists, promoting binding. The importance of the overall glycan structure for substrate binding is further highlighted by previous studies, which showed that the K_m of acceptor peptides fell by about 10-fold when Dol-PP-$GlcNAc_2$-Man_9-Glc_{0-3} instead of Dol-PP-$GlcNAc_2$ was used as the glycosyl donor [14]. Despite Dol-PP-$GlcNac_2$-Man_9-Glc_3 being the natural and preferred substrate, we used Dol-PP-[^{14}C]chitobiose throughout our studies because (i) the ^{14}C-labelled chitobiosyl lipid was accessible in larger quantities and higher purity and (ii) the [^{14}C]glycopeptides synthesized in the OST reaction could be followed and quantified by chloroform/methanol/water phase partition more easily than those possessing larger glycan chains.

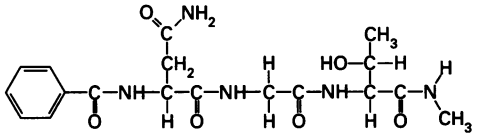


Figure 1 Structure of the *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ acceptor peptide I

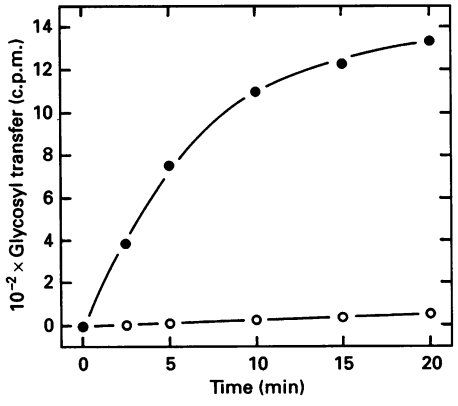


Figure 2 Glycosyl donor properties of Dol-PP-[^{14}C]GlcNAc and Dol-PP-$GlcNAc_2$

Pig liver OST was incubated under standard assay conditions in the presence of peptide I (1 mM) and either 2000 c.p.m. of Dol-PP-[^{14}C]$GlcNAc_2$ (●) or 4000 c.p.m. of Dol-PP-[^{14}C]GlcNAc (○). At given times the reactions were stopped by the addition of 0.5 ml of methanol, and [^{14}C]glycopeptide formation was determined by the phase partition procedure detailed in the Materials and methods section. Transfer rates were not corrected for substrate depletion.

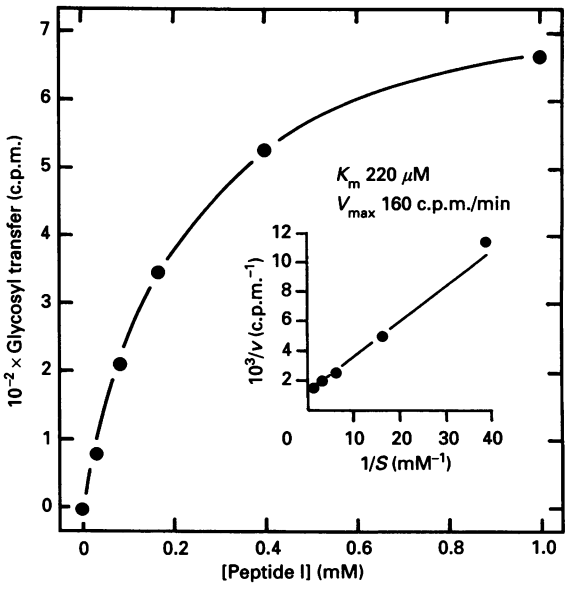
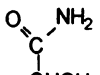
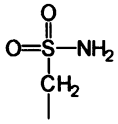
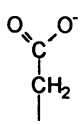
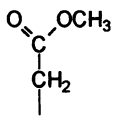
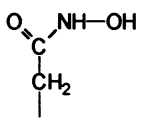
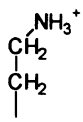
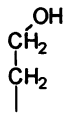


Figure 3 N-Glycosylation of peptide I as a function of peptide concentration

OST was incubated under standard assay conditions with 3000 c.p.m. of Dol-PP-[^{14}C]$GlcNAc_2$ and various concentrations of the acceptor peptide I. The reactions were run for 5 min at 25 °C; they were stopped by the addition of 0.5 ml of methanol, and the methanol phase, containing the [^{14}C]glycopeptides, was processed as described in the Materials and methods section.

Table 1 Structure and acceptor properties of tripeptides containing a modified asparagine side chain

Data for derivatives II and III were calculated assuming a 1:1 mixture of β-hydroxyasparagine enantiomers. n.d., Not determined.

Derivative	Group replacing asparagine in tripeptide I	Structure	Glycosyl acceptor K_m (mM)	Glycosyl acceptor $V_{max.}$ (c.p.m./min)	Glycosyl acceptor $V_{max.}/K_m$	Inhibition of OST
I	N-α-Bz-Asn-Gly-Thr-$NHCH_3$		0.22	160	727	–
II	D/L-β-Hydroxyasparagine (*threo*)		9.5	60	6.3	n.d.
III	D/L-β-Hydroxyasparagine (*erythro*)		11.0	55	5.0	n.d.
IV	L-Cysteinesulphonamide			–		–
V	L-Aspartic acid			–		–
VI	L-Aspartic acid β-methyl ester			–		–
VII	L-Asp-β-hydroxamic acid			–		50% at 20 mM
VIII	L-α,γ-DABA			–		50% at 140 μM
IX	L-α-Amino-γ-hydroxybutyric acid			–		–

Substitution of the asparagine acceptor site by amino acids with chemically modified side chains

Table 1 summarizes the structures of tripeptides in which the side chain functionality of the asparagine residue was replaced by amino acids differing in size, polarity and ionic properties. Derivatives II and III were synthesized from racemic *threo*- and *erythro*-β-hydroxyasparagine respectively and their concentrations in the glycosylation assay were calculated, assuming a 1:1 mixture of β-hydroxyasparagine enantiomers. All other α-amino acids used were of the L-configuration.

Under standard assay conditions derivatives II and III were found to have similar acceptor properties. Their relative glycosylation rates ($V_{max.}/K_m$) were, however, 120- to 150-fold lower than that for peptide I. This difference apparently not only originates from an increase in K_m, but also from a decrease in $V_{max.}$, indicating that the β-hydroxy group in either configuration affects both binding and catalytic parameters (Table 1). The increase in K_m is likely to be steric in origin, whereas

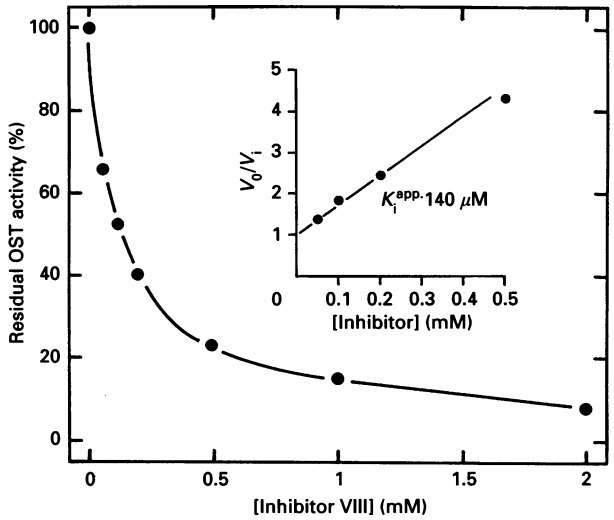


Figure 4 Concentration-dependent inhibition of pig liver OST by the αγ-DABA derivative VIII

Dol-PP-[^{14}C]$GlcNAc_2$ (3000 c.p.m.) was incubated with OST in the presence of 0.5 mM acceptor peptide I and increasing concentrations of the inhibitor VIII. The reactions were run for 10 min at 25 °C and [^{14}C]glycosyl transfer to peptide I was determined as detailed in the Materials and methods section.

the reduction in $V_{max.}$ may be caused by the electron-withdrawing effect of the hydroxy group, decreasing the ability of the β-acid amide to act as a nucleophile in the glycosylation reaction.

Apart from the Asp-hydroxamic acid derivative (VII) which shows weak inhibition of OST activity (50% at 20 mM), tripeptides containing cysteinesulphonamide (IV), aspartic acid (V) and aspartic acid β-methyl ester (VI) neither displayed acceptor properties nor (at 10 M) inhibited glycosylation of peptide I. This shows that these side-chain modifications interfere dramatically with substrate recognition (Table 1). In the case of peptides IV, VI (and VII), binding to OST may be impaired by steric hindrance, whereas in the case of peptide V we assume that the negative charge at the β-carboxyl group is inhibitory.

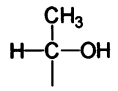
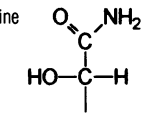
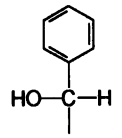
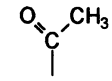
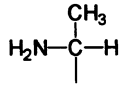
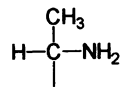
In contrast with these non-acceptor derivatives, the αγ-DABA analogue (VIII), although not acting as an acceptor, was found to inhibit sugar transfer to peptide I efficiently. This observation is consistent with previous studies by Imperiali et al. [6], who used a similar tripeptide but calf liver crude microsomal fraction as the enzyme source. The inhibition by derivative VIII of OST activity was concentration-dependent, with 50% inhibition being observed at ≈ 140 μM (Figure 4). Since the γ-amino group (pK_s > 10) is largely protonated at pH 7.2, the high inhibitory potential of VIII is best explained by ion-pair formation involving the charged β-amino group and an anionic base at the active site of the enzyme. This view is supported by the observation that the inhibitory potential is lost completely when the cationic β-amino group is replaced by an uncharged β-hydroxy group (homoserine; derivative IX) and furthermore provides a plausible explanation of why a negative charge at this particular position (peptide V) is rejected by the enzyme.

Effect of structural modifications to the threonine side chain on binding and glycosylation parameters

Previous studies had shown that substitution of serine for threonine in the Asn-Xaa-Thr/Ser sequence reduced the peptide

Table 2 Substrate properties of tripeptide derivatives containing structurally modified amino acid in the threonine position

K_m and $V_{max.}$ for derivative XV were calculated, assuming a 1:1 mixture of *threo*-α,β-DABA enantiomers. n.d., Not determined.

Derivative	Group replacing threonine in tripeptide I	Structure	Glycosyl acceptor K_m (mM)	$V_{max.}$ (c.p.m./min)	$V_{max.}/K_m$	Inhibition of OST
X	L-Serine		1.4	80	57	–
XI	L-Allothreonine			–		–
XII	D/L-β-Hydroxyasparagine (*threo*)			–		–
XIII	L-Phenylserine			<3% transfer at 10 mM		≈30% at 10 mM
XIV	D/L-α-Amino-β-oxobutyric acid			–		–
XV	D/L-α,β-DABA (*threo*)		5.0	37	6.8	n.d.
XVI	D/L-α,β-DABA (*erythro*)			–		–

K_m severalfold, suggesting a hydrophobic binding pocket for the β-methyl group at the active site of OST [5,14]. In order to obtain further information, we replaced threonine in peptide I by serine (X), allothreonine (XI), *threo*-β-hydroxyasparagine (XII), *threo*-phenylserine (XIII), α-amino-β-oxobutyric acid (XIV), *threo*-α,β-DABA (XV) and *erythro*-αβ-DABA (XVI) (Table 2). The kinetic data obtained with peptide X show that the lack of the β-methyl group increased the K_m value by approx. 6.3-fold in comparison with peptide I, whereas $V_{max.}$ was simultaneously reduced by approximately 2-fold, consistent with previous studies [5,14]. The effect on $V_{max.}$ in particular indicates that the hydroxyamino acid must play an active role in the catalytic process (see under 'Conclusions'). Peptide XI, obtained by replacing threonine with allothreonine, was not glycosylated nor was the glycosylation of peptide I inhibited, revealing that a *threo*-β-C configuration is crucial for substrate recognition. Since the size and electronic properties of threonine and allothreonine are identical, it is reasonable to assume that steric factors are responsible for this discrimination, suggesting in turn that the side chain of threonine, including its β-hydrogen atom, is in close contact with complementary structures at the active site of the enzyme. Recognition by OST is also impaired when the β-methyl group of threonine is replaced by a polar amide group (XII), whereas the phenylserine derivative (XIII), despite having a more bulky side chain and displaying only marginal acceptor properties, inhibits the

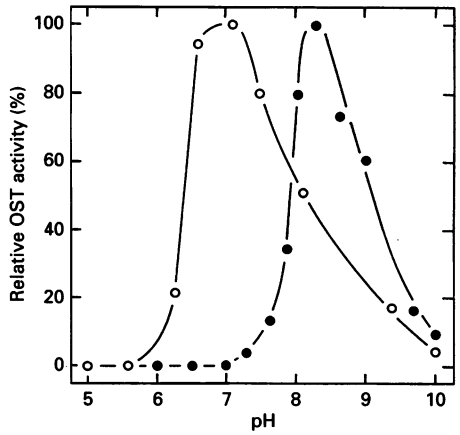


Figure 5 Acceptor properties of peptide I and the αβ-DABA derivative XV as a function of pH

At a given pH, purified pig liver OST was incubated with 3000 c.p.m. of Dol-PP-[^{14}C]GlcNAc$_2$ in the presence of either 0.5 mM peptide I (○) or 10 mM derivative XV (●). After 5 min (peptide I) or 30 min (peptide XV), [^{14}C]glycosyl transfer was assayed as described in the Materials and methods section. For clarity, numbers are normalized on maximal transfer rates (100%).

glycosylation of peptide I by about 30% at 10 mM. These cumulative data support the view of a specific β-methyl group interaction, which does not occur with larger and polar substituents.

Owing to its ability to act as a hydrogen-bond acceptor for the β-amide proton of asparagine, the β-keto derivative (XIV) was expected to be bound to, but not glycosylated by, OST. It was synthesized by oxidation of the β-hydroxy group in the non-acceptor peptide XI. This strategy was chosen because α-amino-β-keto acids are chemically unstable. The structure of derivative XIV, being consistent with NMR and MS data, was confirmed further by reduction of the planar β-keto group to the alcohol, yielding a product mixture with glycosyl-acceptor properties. This is consistent with borohydride reduction being non-stereospecific, thus resulting in the formation of both *threo* (peptide I) and *erythro* (peptide XI) diastereomers. Enzymic measurements with derivative XIV showed, however, that the planar keto derivative was not inhibitory nor could it be glycosylated. This observation indicates that a tetrahedral configuration at the β-C atom is essential for substrate recognition, and that alterations affecting this configuration are not tolerated by OST.

In order to substantiate further the role of the β-hydroxy group in the catalytic process, we synthesized two tripeptides containing either racemic *threo*-α,β- (XV) or *erythro*-α,β-DABA (XVI) in place of threonine. Acceptor studies revealed that analogue XV displayed glycosyl-acceptor properties, whereas, under identical incubation conditions, analogue XVI did not. This observation is consistent with the acceptor/non-acceptor properties of peptides I and XI respectively again underlining the importance of the *threo* configuration for substrate recognition. It also shows that the amino group is able to mimic the hydroxy group in the glycosylation process. This is not altogether surprising since the hydroxy group and the NH_2 group are of similar polarity and both can form hydrogen bonds. As seen in Figure 5, the pH profile for the glycosylation of analogue XV is shifted by approximately +1 pH unit from that for peptide I. Since the pK_s of the β-amino group is close to 7.9, this pH shift is best explained by the assumption that the non-protonated

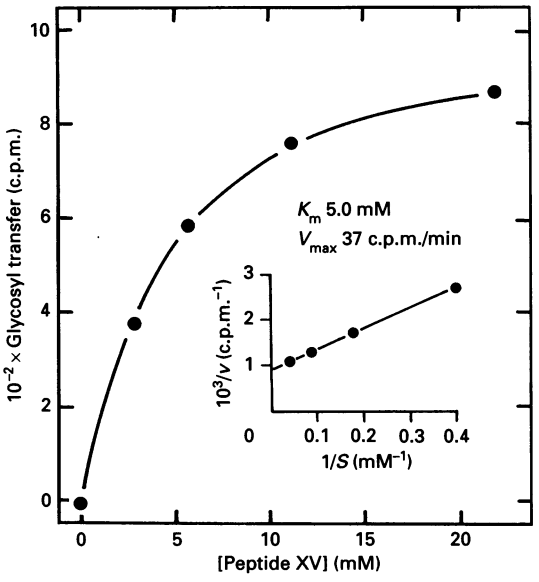


Figure 6 Glycosylation of derivative XV as a function of peptide concentration

Increasing amounts of analogue XV were incubated at pH 8.2 (pH optimum of transfer, see Figure 5) with purified OST and 3000 c.p.m. of Dol-PP-[^{14}C]GlcNAc$_2$. After 30 min at 25 °C [^{14}C]glycosyl transfer was assayed as described in the Materials and methods section. The peptide concentrations given represent half the total concentration of derivative XV in the assay, assuming that the D-enantiomer lacks acceptor or inhibitory properties.

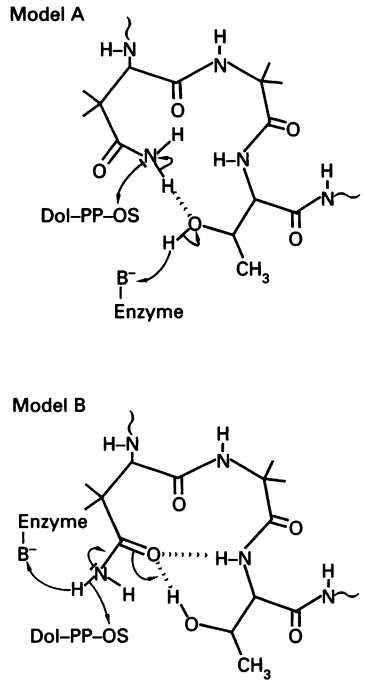


Figure 7 Proposed mechanisms describing the functional role of the hydroxy side chain in Asn-Xaa-Thr/Ser during catalysis

Model A was proposed by Bause and Legler [5], and model B is adapted from that of Imperiali et al. [6]. For clarity the polypeptide backbone in both models is drawn schematically in the same orientation. It should be noted, however, that hydrogen-bonding as in model A is particularly favoured in β-turn or other loop structures, whereas the interaction shown in model B requires an Asx-turn conformation at the glycosylation site. OS, Glc_3-Man_9-$GlcNAc_2$ oligosaccharide.

rather than the charged amino group is the reactive species, overtaking the catalytic role of the hydroxy group in the glycosylation step. This in turn implies that the ability of the β-NH_2/β-OH group to function as a hydrogen-bond acceptor is necessary for catalysis. At pH 8.2 (the optimum for glycopeptide formation), the glycosylation of analogue XV showed saturation kinetics with a K_m of ≈ 5.0 mM (calculated for a D/L ratio of 1:1, and assuming that the D-isomer is inactive as acceptor) and a $V_{max.}$ of ≈ 37 c.p.m./min (Figure 6). The relative glycosylation rate estimated from these data is about 100-fold lower than $V_{max.}/K_m$ for peptide I. It should be noted, however, that the acceptor measurements were carried out at pH 8.2, a pH at which the catalytic activity of OST is less than 50 % of that at the pH optimum of the enzyme (Figure 5).

Conclusions

The N-glycosylation of asparagine probably occurs by simple nucleophilic attack by the β-amide electron pair on the C-1 atom of the phosphoacetal-activated Dol-PP-oligosaccharide. Since amides are known to be weakly nucleophilic, it is intriguing to speculate on how the β-nitrogen acquires sufficient nucleophilicity. Two catalytic models dealing with this aspect are currently under discussion (Figure 7). Model A, previously suggested by us [5], depends on the assumption that the nucleophilicity of the β-amide nitrogen is enhanced by hydrogen-bonding with the β-amide of asparagine as the hydrogen-bond donor and the threonine/serine hydroxy group as the acceptor. During glycosylation, the –N–**H**···O– hydrogen is assumed to be transferred to the β-hydroxy group from which a proton is simultaneously delivered to an appropriate base at the active site of the enzyme. Thus the β-hydroxy group would accomplish two functions: (i) it would enhance the β-amide nucleophilicity and (ii) it would act as a 'proton vehicle' for the β-amide hydrogen to be replaced by the carbohydrate chain.

An alternative model (model B in Figure 7), proposed by Imperiali et al. [6], postulates that a proton dissociates from the β-nitrogen generating an imidate structure, which acts as a competent nucleophile in the glycosylation reaction. Proton dissociation is assumed to be enhanced by hydrogen-bonding involving the β-carbonyl group of asparagine as the acceptor and both the β-OH and the α-NH group of threonine/serine as donors. These interactions would be favoured by Asx-turn formation at the glycosylation site. However, as the acidity of acid amines ($pK_A > 15$) is extremely low [24], it is questionable whether hydrogen-bonding is energetically capable of increasing K_A for the β-amide sufficiently to release a proton to a complementary base.

Although the bulk of experimental data available at present is not inconsistent with either model, there are several lines of evidence favouring model A. (i) The observed decrease in $V_{max.}$ measured for the β-hydroxyasparagine derivatives II and III can be explained plausibly by β-amide nucleophilicity being reduced by the electron-withdrawing effect of the hydroxy group. This interpretation is supported by the observation that β-fluoroasparagine-containing tripeptides, although not glycosylated, are still inhibitory [25]. Furthermore, studies with particulate cell-free translation systems show that the N-glycosylation of newly synthesized polypeptides is efficiently blocked when β-fluoroasparagine is incorporated instead of

asparagine [26,27]. The effect of the fluoro substituent, in line with model A, is clearly in contradiction to model B, because in the latter the electron-withdrawing properties of the fluoro group would be expected to promote proton dissociation and thus favour imidate formation. (ii) The pH-dependence measured for the glycosylation of peptide I and the *threo*-α,β-DABA derivative (XV) shows that the non-protonated β-amino group mimics the catalytic function of the threonine hydroxy group; this indicates that the NH_2 group may act as a hydrogen-bond acceptor rather than as a donor, in accordance with mechanism A. By contrast, an 'inverted' hydrogen-bonding interaction, which would obviously assist the dissociation of the amide proton (model B), may also be feasible with the charged NH_3^+ group. It cannot be excluded, however, that, owing to steric hindrance, binding of the cationic peptide may be prevented in this case. (iii) Previous studies showed that OST activity is inhibited irreversibly by peptide derivatives containing epoxyvinylglycine in the threonine position [28]. Most importantly, inactivation was found to be closely associated with the glycosylation process. Based on kinetic data [28] and recent double-labelling experiments (E. Bause, W. Breuer and M. Wesemann, unpublished work) a suicide mechanism of inactivation seems likely, resulting in the glycosylation of the inhibitor peptide, while the epoxy function, activated by accepting an amide proton, simultaneously alkylates a base at the active site. This 'suicide' inactivation is in apparent contradiction to model B, because hydrogen-bonding between the asparagine side chain and the epoxy function, assisting inhibitor glycosylation as well as promoting the alkylation step by protonation of the oxiran ring, is possible in model A but not in model B.

The strong inhibitory potential at pH 7.0 of derivative VIII, in which the β-amide group is converted into a primary alkylamine, shows that the protonated amino group of this derivative may interact with a negatively charged base at the active site of OST. This interpretation is consistent with the observation that a negative charge at the β-C atom (peptide V) is inhibitory. The strength of the ionic interaction is apparently so large that it can compensate for the lack of the β-carbonyl group, as indicated by an inhibition constant that is of the same order of magnitude as the K_m value of acceptor peptide I. The contribution of both the β-carbonyl and the cationic $-NH_3^+$ group to peptide and inhibitor binding respectively is further highlighted by the observation that the homoserine derivative (IX), differing from VIII in having a neutral hydroxy group, is neither glycosylated nor acts as a competitive inhibitor.

The lack of substrate recognition by OST resulting from the replacement of asparagine by cysteinesulphonamide (IV), aspartic acid β-methylester (VI) or Asp-hydroxyamic acid (VII) shows that substituents larger than the β-acid amide acceptor function do not apparently fit into the corresponding binding site, or only badly. This implies that the acceptor function is in close contact with active-site structures. Spatial constraints appear to be somewhat less pronounced for the β-C position of the asparagine side chain as indicated by the moderate acceptor properties of both the *erythro*- and *threo*-β-hydroxyasparagine derivatives (II and III). The glycosylation of these derivatives furthermore supports the view that the inability of OST to glycosylate β-fluoroasparagine-containing polypeptides [25,26] is caused by the electron-withdrawing properties of the β-fluoro and β-hydroxy groups rather than by steric effects.

The enzymic measurements with the peptide derivatives modified in the side-chain structure of threonine confirm that a specific binding site for the β-methyl group must exist, into which more bulky substituents do not fit or fit only badly, independently of whether they are polar (XII) or hydrophobic (XIII). Substrate recognition by OST is also impaired by inversion of the configuration at the β-C atom of the hydroxy amino acid (XI, XVI) or by conversion of its tetrahedral configuration into a planar one (XIV). These data show that not only the β-acid amide acceptor site but also the side chain substituents of the hydroxyamino acid in the Asn-Xaa-Thr/Ser motif must be involved with the active site of OST, thus explaining the unique specificity of the enzyme with regards to both substrate binding and catalytic properties.

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REFERENCES

1 Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. **54**, 631–664
2 Bause, E. (1979) FEBS Lett. **103**, 296–299
3 Welply, J. K., Shenbagamurthi, P., Lennarz, W. J. and Naider, F. (1983) J. Biol. Chem. **258**, 11856–11863
4 Kaplan, H. A., Welply, J. K. and Lennarz, W. J. (1987) Biochim. Biophys. Acta **906**, 161–173
5 Bause, E. and Legler, G. (1981) Biochem. J. **195**, 639–644
6 Imperiali, B., Shannon, K. L., Unno, M. and Rickett, K. W. (1992) J. Am. Chem. Soc. **114**, 7944–7945
7 Bause, E., Hettkamp, H. and Legler, G. (1982) Biochem. J. **203**, 761–768
8 Bause, E. (1983) Biochem. J. **209**, 331–336
9 Abbadi, A., Mcharfi, M., Aubry, A., Prémilat, S., Boussard, G. and Marrand, M. (1991) J. Am. Chem. Soc. **113**, 2729–2735
10 Imperiali, G. and Shannon, K. L. (1991) Biochemistry **30**, 4374–4380
11 Struck, D. K., Lennarz, W. J. and Brew, K. (1978) J. Biol. Chem. **253**, 5786–5794
12 Bause, E. and Hettkamp, H. (1979) FEBS Lett. **108**, 341–344
13 Gavel, Y. and von Heijne, G. (1990) Protein Engineering **3**, 433–442
14 Breuer, W. and Bause, E. (1995) Eur. J. Biochem. **228**, 689–696
15 Bodanszky, M. and Bodanszky, A. (1984). The Practice of Peptide Synthesis, Springer-Verlag, Berlin and Heidelberg
16 Houben-Weyl, H. (1974) Methoden der Organischen Chemie, vol. 15, Thieme-Verlag, Stuttgart
17 Jones, C. W., Leyden, D. E. and Stammer, C. H. (1969) Can. J. Chem. **47**, 4363–4366
18 Liwschitz, Y. and Singerman, A. (1967) J Chem. Soc. (C) 1696–1700
19 Albright, J. D. and Goldman, L. (1967) J. Am. Chem. Soc. **89**, 2416–2423
20 Borch, R. F., Berbstein, M. D. and Durst, H. D. (1971) J. Am. Chem. Soc. **93**, 2897–2904
21 Bray, G. A. (1970) Anal. Biochem. **1**, 279–285
22 Bause, E. and Lehle, L. (1979) Eur. J. Biochem. **101**, 531–540
23 Ronin, C. (1980) FEBS Lett. **113**, 340–344
24 Bordwell, F. G. (1988) Acc. Chem. Res. **21**, 456–463
25 Rathod, P. K., Tashjian, A. H. and Abeles, R. H. (1986) J. Biol. Chem. **261**, 6461–6469
26 Hortin, G., Stern, A. M., Miller, B., Abeles, R. H. and Boime, J. (1983) J. Biol. Chem. **258**, 4047–4050
27 Tillmann, U., Günther, R., Schweden, J. and Bause, E. (1987) Eur. J. Biochem. **162**, 65–642
28 Bause, E. (1983) Biochem. J. **209**, 323–330

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