

# Oligosaccharyltransferase is highly specific for the hydroxy amino acid in Asn-Xaa-Thr/Ser

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**Abstract** Pig liver oligosaccharyltransferase (OST), which is involved in the en bloc transfer of the Dol-PP-linked GlcNAc<sub>2</sub>-Man<sub>9</sub>-Glc<sub>3</sub> precursor on to asparagine residues in the Asn-Xaa-Thr/Ser sequence, is highly stereospecific for the conformation of the 3-carbon atom in the hydroxy amino acid. Moreover, substitution of the hydroxy group by either SH as in cysteine, or NH<sub>2</sub> as in  $\beta,\gamma$ -diamino-butanoic acid as reported previously [Bause, E. et al., *Biochem. J.* 312 (1995) 979–985], followed by the determination of the pH optimum for enzymatic activity, indicates that neither a negative nor a positive charge in the hydroxy amino acid position is tolerated by the enzyme. Binding of the threonine  $\beta$ -methyl group by OST is also specific, with serine, L-threo- $\beta$ -hydroxynorvaline and L- $\beta$ -hydroxynorleucine containing tripeptides all bound much less efficiently than the threonine peptide itself. The data are interpreted in terms of a highly stereospecific hydrophobic binding pocket for the threonine CH<sub>3</sub>-CH(OH) group. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Oligosaccharyltransferase; Stereospecificity; Threonine analogue; Hydrophobic binding site; Enzyme kinetics

## 1. Introduction

Oligosaccharyltransferase (OST; EC 2.4.1.119) is a hetero-oligomeric protein complex associated with the endoplasmic reticulum membrane, which catalyses the en bloc transfer of GlcNAc<sub>2</sub>-Man<sub>9</sub>-Glc<sub>3</sub> from Dol-PP on to specific asparagine residues of the nascent polypeptide chain [1]. A prerequisite for this transfer reaction is that the acceptor asparagine forms part of an Asn-Xaa-Thr/Ser triplet [2–4]. Due to the specificity of OST, its activity can be measured reliably in crude microsomal fractions, or even total cell extracts, using synthetic Asn-Xaa-Thr/Ser peptides as acceptors and Dol-PP-oligosaccharides as glycosyl donors; lipid-linked chitobiose (GlcNAc<sub>2</sub>) is the shortest glycan that can be transferred by the enzyme [5]. This technique greatly facilitated studies on the characterisation of the substrate specificity of the enzyme, even before it had proved possible to purify the enzyme protein, providing important insights into the mechanism of the transglycosylation reaction and demonstrating that the hydroxy amino acid

in the consensus sequence is required for catalysis [6–9]. Thus, it was shown that any other amino acid within or adjacent to the consensus sequence is tolerated, except for a proline residue in the Xaa position or on the C-terminal side of the consensus sequence, either of which are not compatible with asparagine glycosylation, although the kinetics of glycosylation differed depending on the substituted amino acid [10,11].

In this paper we report on the synthesis and glycosylation properties of tripeptide substrates in which the hydroxy amino acid in the Asn-Xaa-Thr/Ser triplet had been replaced by other amino acids, including non-physiological analogues of threonine. Our results point to a specific binding pocket for the  $\beta$ -methyl group of threonine. They show, furthermore, that the *R*-configuration at  $\beta$ -C atom of the hydroxy amino acid is crucial for OST recognition, supporting the view that not only the  $\beta$ -methyl group, but also the  $\beta$ -hydrogen must be in close contact with the active site of the enzyme.

## 2. Materials and methods

### 2.1. Materials

Materials and chemicals were obtained from the following sources: UDP-*N*-acetyl-[<sup>14</sup>C]glucosamine, specific radioactivity 300 mCi/mmol (Amersham Pharmacia); free amino acids, L-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline, *N,N'*-dicyclohexylcarbodiimide, benzoylchloride, Triton X-100 and acylase I from porcine kidney (Fluka); trifluoroacetic acid and silica gel G 60 plates (Merck); *N*-BOC and *N*-CBZ-L amino acid derivatives (Bachem); Dol-P (Sigma). All other chemicals used were of analytical grade.

### 2.2. Determination of OST activity

Standard incubation mixtures for measuring peptide glycosylation contained in a total volume of 100  $\mu$ l: 50 mM Tris-HCl, pH 7.2, 10 mM MnCl<sub>2</sub>, 0.8% Triton X-100, 150 mM sodium acetate, 0.3 M sucrose, 5000 cpm of Dol-PP-[<sup>14</sup>C]GlcNAc<sub>2</sub>, and peptide. Due to the relatively low solubility of the tripeptides in water, stock solutions in dimethyl sulphoxide (DMSO)/water (30:70 v/v) were prepared, giving 10% (v/v) DMSO in the final incubation mixture. Each reaction was started by the addition of pig liver microsomes and maintained at 25°C. After given times, the reaction was stopped by the addition of 0.5 ml of methanol, followed by centrifugation of the suspension. The supernatant was removed and 0.75 ml chloroform plus 0.15 ml water added, producing two phases. [<sup>14</sup>C]Glycopeptides present in the aqueous upper phase were then determined by liquid scintillation counting [9]. *V*<sub>max</sub> values, given as cpm, represent relative values determined under identical reaction conditions and using the same enzyme preparation.

### 2.3. Peptide synthesis

All peptide derivatives were synthesised in solution by adapting published procedures and blocking strategies, using L-amino acids unless otherwise stated [9,12]. D/L-threo/erythro- $\beta$ -Hydroxynorvaline (Hnv) and D/L-threo/erythro- $\beta$ -hydroxynorleucine (Hnl) were synthesised as described [13]. Separation of the D- and L-isomers of Hnv and Hnl was performed by acylase I treatment of the racemate after *N*-

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**Abbreviations:** Hnv, hydroxynorvaline; Hnl, hydroxynorleucine; OST, oligosaccharyltransferase

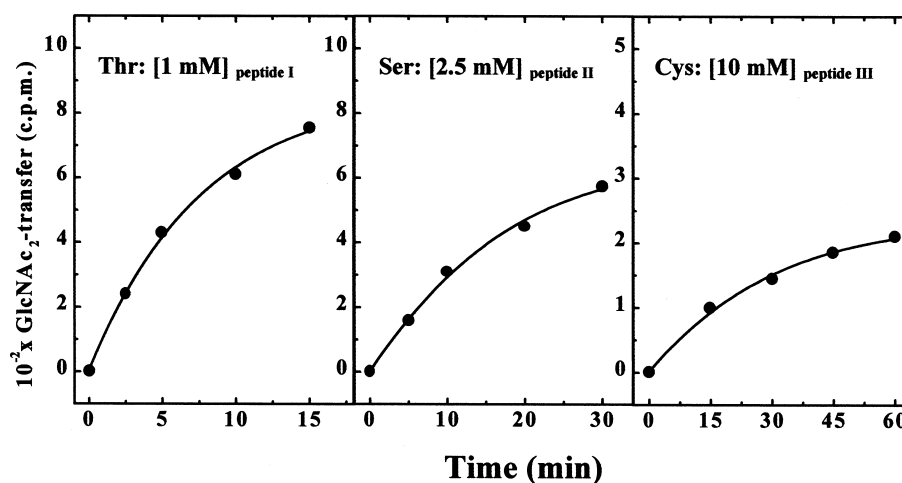


Fig. 1. Glycosylation kinetics for *N*-benzoyl-Asn-Gly-Thr-NHCH<sub>3</sub> (peptide I), *N*-benzoyl-Asn-Gly-Ser-NHCH<sub>3</sub> (peptide II) and *N*-benzoyl-Asn-Gly-Cys-NH<sub>2</sub> (peptide III). Reaction conditions were as described in the text.

acetylation [14]. Diastereomeric tripeptides containing *L*-threolerythro-Hnv were separated by preparative silica gel chromatography using chloroform/MeOH (7:3 v/v) as the solvent. The purity of the tripeptides was checked by thin layer chromatography on silica gel G 60 plates using different solvent systems (*n*-butanol/acetic acid/water, 4:1:1 v/v; chloroform/methanol/acetic acid, 65:25:5 v/v and chloroform/water, 7:3 v/v). The structure of the peptide derivatives was confirmed by <sup>1</sup>H-nuclear magnetic resonance (NMR) and mass spectrometry (MS).

***N*-Benzoyl-Asn-Gly-(2S,3R)-Thr-NHCH<sub>3</sub> (I):** *m/z* 408 (MH<sup>+</sup>); δ 1.0 (d; CH<sub>3</sub>, Thr); 2.55 (d; CH<sub>3</sub>, -NHCH<sub>3</sub>); 2.65 (d; CH<sub>2</sub>, Asn); 3.8 (m; CH<sub>2</sub>, Gly); 4.05–4.1 (m; C<sub>α</sub>H, Thr; C<sub>β</sub>H, Thr); 4.75 (m; CH, Asn); 4.8 (d; OH, Thr); 6.9+7.45 (2 s; NH<sub>2</sub>, Asn); 7.5–7.9 (m; C<sub>6</sub>H<sub>5</sub>); 7.65 (m; NH, Thr; NH, -NHCH<sub>3</sub>); 8.25 (t; NH, Gly); 8.75 (d; NH, Asn).

Molecular masses of the stereoisomeric derivatives and selected <sup>1</sup>H-NMR data were as follows.

***N*-Benzoyl-Asn-Gly-Ser-NHCH<sub>3</sub> (II):** *m/z* 394 (MH<sup>+</sup>); δ 3.6 (t; CH<sub>2</sub>, Ser); 4.2 (m; CH, Ser); 4.8 (t; OH, Ser); 7.8 (d; NH, Ser).

***N*-Benzoyl-Asn-Gly-Cys-NH<sub>2</sub> (III):** *m/z* 396 (MH<sup>+</sup>); δ 2.25 (t; SH, Cys); 2.8 (m; CH<sub>2</sub>, Cys); 4.3 (m; CH, Cys); 7.9 (d; NH, Cys).

***N*-Benzoyl-Asn-Gly-(2S,3R)-L-threo-Hnv-NHCH<sub>3</sub> (IV):** *m/z* 444 (MNa<sup>+</sup>); δ 0.8 (m; CH<sub>3</sub>, L-Hnv); 1.3 (m; CH<sub>2</sub>, L-Hnv); 3.75 (m; C<sub>β</sub>H, L-Hnv); 4.15 (C<sub>α</sub>H, L-Hnv); 4.75 (m; OH, L-Hnv); 7.75 (m; NH, L-Hnv).

***N*-Benzoyl-Asn-Gly-(2R,3S)-D-threo-Thr-NHCH<sub>3</sub> (VI):** *m/z* 408 (MH<sup>+</sup>); δ 1.0 (d; CH<sub>3</sub>, D-Thr); 4.05–4.1 (m; C<sub>α</sub>H, D-Thr; C<sub>β</sub>H, D-Thr); 4.8 (d; OH, D-Thr); 7.65 (m; NH, D-Thr).

***N*-Benzoyl-Asn-Gly-(2S,3S)-L-allo-Thr-NHCH<sub>3</sub> (VII):** *m/z* 408 (MH<sup>+</sup>); δ 1.0 (d; CH<sub>3</sub>, L-allo-Thr); 3.85 (m; C<sub>β</sub>H, L-allo-Thr); 4.1 (m; C<sub>α</sub>H, L-allo-Thr); 4.85 (m; OH, L-allo-Thr); 7.75 (d; NH, L-allo-Thr).

***N*-Benzoyl-Asn-Gly-(2R,3R)-D-allo-Thr-NHCH<sub>3</sub> (VIII):** *m/z* 408 (MH<sup>+</sup>); δ 1.0 (d; CH<sub>3</sub>, D-allo-Thr); 3.85 (m; C<sub>β</sub>H, D-allo-Thr); 4.1 (m; C<sub>α</sub>H, D-allo-Thr); 4.9 (d; OH, D-allo-Thr); 7.8 (d; NH, D-allo-Thr).

#### 2.4. General methods

Dol-PP-[<sup>14</sup>C]GlcNAc<sub>2</sub> was prepared as described in [11], and pig liver microsomes as described in [15]. Radioactivity was determined by liquid-scintillation counting using Bray's solution as counting fluid [16]. The molecular mass of peptides (MH<sup>+</sup>) was determined on a VG ZAB FAB mass spectrometer; <sup>1</sup>H-NMR data were measured in [<sup>2</sup>H]DMSO using a Bruker AMX-500 MHz spectrometer.

### 3. Results and discussion

#### 3.1. The structure of the hydroxy amino acid in

*Asn-Xaa-Thr/Ser affects both binding and catalysis*

Under in vitro conditions, OST binds and glycosylates tri-

peptides containing the Asn-Xaa-Thr/Ser sequence so long as their N- and C-termini are blocked by amide formation [11,17]. Since tripeptide derivatives are easily accessible by chemical synthesis, their use as OST substrates is a convenient approach for obtaining structural information on the specificity and catalytic properties of the enzyme [7,9]. Extending our studies on the functional significance of the hydroxy amino acid in the consensus sequence, we have synthesised a series of tripeptide substrates which contained, in place of threonine in the *N*-benzoyl-Asn-Gly-Thr-NHCH<sub>3</sub> peptide (I), either serine, cysteine or various threonine analogues. The consequences of these structural modifications on the OST catalysed reaction were then analysed by a standard glycosylation assay, using \*Dol-PP-[<sup>14</sup>C]GlcNAc<sub>2</sub> as the glycosyl donor and pig liver crude microsomes as the enzyme source. All tripeptide derivatives were prepared in solution following standard methods of protein chemistry and characterised by MS and NMR.

The kinetics shown in Fig. 1 indicate that both the *N*-benzoyl-Asn-Gly-Thr-NHCH<sub>3</sub> (I), as well as the *N*-benzoyl-Asn-Gly-Ser-NHCH<sub>3</sub> (II) tripeptide, are potent glycosyl acceptors in the OST catalysed reaction, under the standard assay conditions used. The observed deviation from linearity of glycosyl

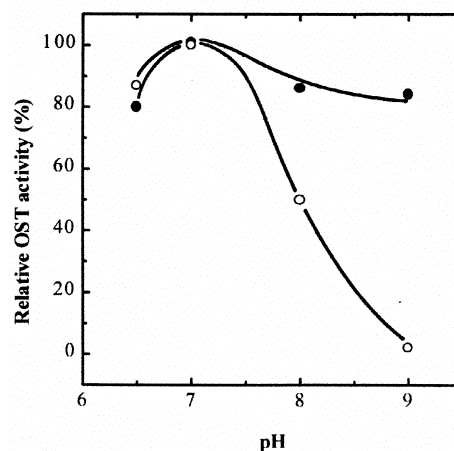


Fig. 2. Relative OST glycosylation activity versus pH for Thr peptide (I) and Cys peptide (III). Incubation time was 15 min for peptide I (●) and 60 min for peptide III (○).

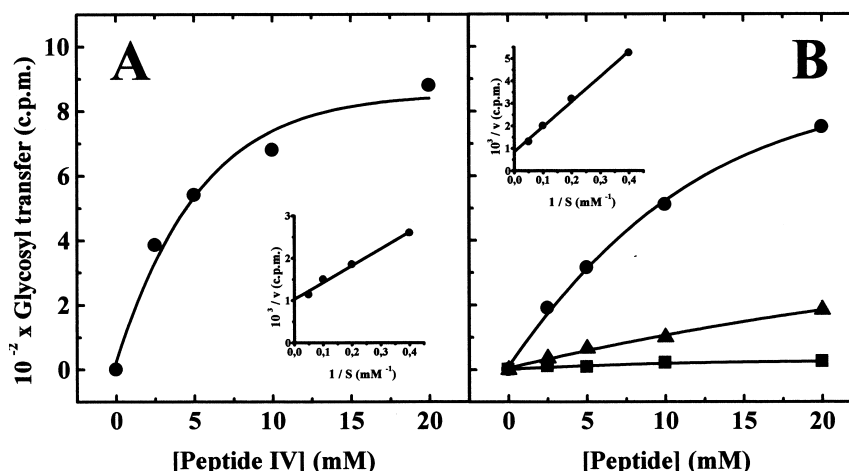


Fig. 3. Glycosylation rate versus substrate concentration for (A) the *L*-threo-Hnv peptide IV and (B) for the diastereomeric *D*-Thr peptide (VI) (■), *L*-allo-Thr peptide (VII) (▲), and *D*-allo-Thr peptide (VIII) (●). Lineweaver-Burk plots are shown inset for peptides IV and VIII. Incubation time was 60 min in all cases.

transfer at longer incubation times is most likely due to Dol-PP-[ $^{14}\text{C}$ ]GlcNAc<sub>2</sub> depletion for which the numbers have been not corrected. For peptide I values of  $K_M \approx 0.16$  mM and  $V_{\max} = 60$  cpm could be obtained from the Lineweaver-Burk plot, whereas substitution of threonine by serine (peptide II) was found to bring about an approximately four-fold increase in  $K_M$  and two-fold reduction of  $V_{\max}$  (Table 1). This indicates that substrate binding as well as catalytic rate are enhanced by a  $\beta$ -methyl group. The lower  $K_M$  for peptide I, compared to peptide II, is best explained by assuming that the  $\beta$ -methyl group binds to a hydrophobic pocket at the active site OST. The difference in  $V_{\max}$ , on the other hand, may be due to electronic effects exerted by the  $\beta$ -methyl group on the  $\beta$ -hydroxy group, whose interaction by hydrogen bonding with the asparagine  $\beta$ -amide group was previously postulated to be required for catalysis [6–9].

In order to characterise further the role of the  $\beta$ -hydroxy group in catalysis, a tripeptide was synthesised which contained cysteine in place of serine. The *N*-benzoyl-Asn-Gly-Cys-NH<sub>2</sub> peptide (peptide III) was found to be glycosylated by OST in a time- (Fig. 1) and concentration-dependent manner (not shown). Its relative glycosylation rate estimated from the initial slope of the concentration dependence was, however, more than 45-fold lower compared with the serine analogue (peptide II). The reduced glycosylation rate for peptide III appears to be caused mainly by an increase in  $K_M$  ( $>15$  mM), indicating a reduction in binding probably caused by both steric and other factors such as weaker hydrogen bond-

ing. Although the Cys peptide (III) was a rather poor acceptor, glycosylation of this peptide was found to be pH-dependent with an optimum at pH 7.2 (Fig. 2) and a steep decrease in enzymatic activity at higher pH, which was not observed when peptide I was used as the acceptor. This observation points to a negative charge in this position, resulting from dissociation of the cysteine SH group ( $pK_a \approx 8.5$ ), not being tolerated by the enzyme.

### 3.2. Binding of the $\beta$ -methyl group is specific

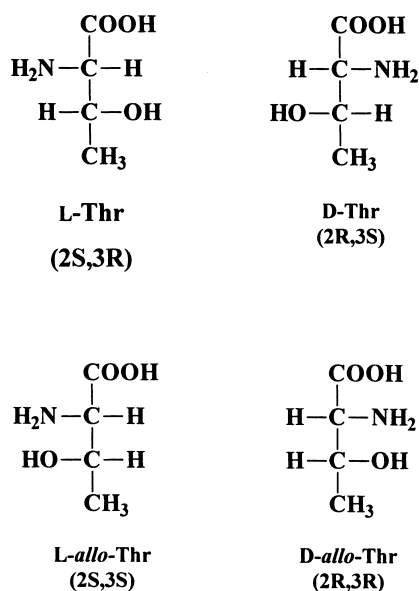
In order to investigate the nature of the proposed  $\beta$ -methyl binding pocket at the active site of OST, two tripeptide derivatives were synthesised containing either *L*-threo- $\beta$ -Hnv (peptide IV) or *L*- $\beta$ -Hnl (peptide V) instead of threonine. Acceptor measurements using peptide IV revealed a time- and concentration-dependent glycosylation with  $K_M \approx 4.3$  mM and  $V_{\max} \approx 17$  cpm (Fig. 3A). Thus, the relative glycosylation rate of peptide IV (given as  $V_{\max}/K_M$ ; see Table 1) was  $\sim 94$ -fold lower compared with the threonine analogue peptide I. The increase in  $K_M$  indicates that the bulkier  $\beta$ -CH<sub>2</sub>-CH<sub>3</sub> group is not able to bind properly, probably due to the restricted size of the hydrophobic pocket. Ineffective binding may, in turn, be the reason for the  $\sim 3.5$ -fold decrease in  $V_{\max}$ , with loss of optimal hydrogen bonding between the  $\beta$ -hydroxy group and the asparagine side-chain, postulated as being essential for catalytic activity. Replacement of the threonine  $\beta$ -methyl by a propyl group (peptide V) was accompanied by a further  $\sim$ eight-fold reduction in the relative glyco-

Table 1  
Kinetic parameters for the *N*-benzoyl-Asn-Gly-Xaa-NHCH<sub>3</sub> tripeptides as oligosaccharyl acceptors for the OST enzyme

Peptide	Xaa	$K_M$ (mM)	$V_{\max}$ (cpm)	$V_{\max}/K_M$ (cpm/mM)	Reduction in rate (x-fold)
I	Thr	$\sim 0.16$	$\sim 60$	$\sim 375$	1 (reference)
II	Ser	$\sim 0.6$	$\sim 28$	$\sim 47$	$\sim 8$
III	Cys-NH <sub>2</sub>	—	—	$\sim 1^a$	$\sim 375$
IV	<i>L</i> -threo-Hnv	$\sim 4.3$	$\sim 17$	$\sim 4$	$\sim 94$
V <sup>b</sup>	<i>L</i> -Hnl	—	—	$\sim 0.5^a$	$\sim 750$
VI	<i>D</i> -Thr	—	—	$\sim 0.025^a$	$\geq 15\,000$
VII	<i>L</i> -allo-Thr	—	—	$\sim 0.25^a$	$\sim 1500$
VIII	<i>D</i> -allo-Thr	$\sim 12.5$	$\sim 19$	$\sim 1.52$	$\sim 245$

<sup>a</sup>Estimated from initial transfer rates.

<sup>b</sup>Contains a  $\sim 2:1$  mixture of *L*-threo/*allo*-isomers.



Scheme 1.

sylation rate compared with the Hnv derivative (peptide IV) (see Table 1). We conclude from these observations that the functional size of the hydrophobic binding pocket at the active site of OST is limited to that of a methyl group. Furthermore, there does not appear to be any additional space available for binding alkyl chains which are bulkier than a methyl group, as in peptide IV and peptide V.

### 3.3. The 3R configuration is crucial for peptide binding

Several stereoisomeric tripeptides were synthesised by replacing threonine (2S,3R) in peptide I with either D-threonine (2R,3S; peptide VI), L-allo-threonine (2S,3S; peptide VII) or D-allo-threonine (2R,3R; peptide VIII) as shown below, in order to study the influence of the  $\alpha$ -C atom and  $\beta$ -C atom configuration in the hydroxy amino acid on peptide binding. Acceptor measurements with these non-physiological substrate analogues showed that inversion of configuration at both the  $\alpha$ - and  $\beta$ -C atoms (peptide VI) results in complete loss of binding and, as a consequence, glycosylation. Inversion at only the  $\beta$ -C atom (peptide VII) was less deleterious, although the acceptor properties of peptide VII were still much lower than for peptide I (Fig. 3B). Replacement of threonine in peptide I by D-allo-threonine, differing from peptide I only in the configuration at the  $\alpha$ -C atom, gave rise to peptide VIII with markedly increased acceptor properties compared with peptide VI or peptide VII, although relative glycosylation ( $V_{\max}/K_M$ ) was still  $\sim 245$ -fold lower compared with that of the threonine analogue peptide I (Fig. 3B). This factor resulted mainly from a  $\sim 78$ -fold increase in  $K_M$  ( $\sim 12.5$  mM) and a  $\sim 3.2$ -fold reduction in  $V_{\max}$ , compared with the threonine peptide I, but only  $\sim 1.5$ -fold compared with the serine peptide II (Table 1). These data show that inversion of the configuration of the  $\alpha$ -carbon atom (from *S* to *R*) is less significant than inversion at the  $\beta$ -C atom, indicating that the *R* configuration at the  $\beta$ -C atom, as in threonine, represents a dominant structural feature for peptide recognition and binding by OST. In turn, it can be concluded

from these observations that not only must the  $\beta$ -methyl group in peptide I be in close contact with the active site of the enzyme, but also so must the  $\beta$ -hydrogen atom (Scheme 1).

### 3.4. Conclusions

The data reported here clearly show that OST activity drops off at high pH, especially when cysteine is substituted for threonine in the acceptor peptide, suggesting that a negative charge at this position blocks enzyme activity. Previous data, using the 3-amino analogue of threonine (L-threo-2,3-diamino-butanoic acid), demonstrated a positive shift of the pH optimum, i.e. to higher pH, indicating that the amino group must be deprotonated for glycosylation to occur [9]. Taken together with our data for the cysteine-substituted peptide, these findings suggest that the OST enzyme cannot function properly with either a positive or negative charge in the side-chain of the amino acid at the Thr/Ser position.

There is strict specificity for the (3R)-CH<sub>3</sub>CH(OH) group in the four threonine analogues studied. Compared to the peptide containing (2S,3R)-threonine, that containing (2R,3R)-D-allo-threonine (peptide VIII; 245-fold reduction) is considerably better than either the peptide with (2S,3S)-L-allo-threonine (peptide VII; 1500-fold reduction) or (2R,3S)-D-threonine, which is hardly glycosylated at all (peptide VI; 15000-fold reduction). Thus, the requirement for the correct configuration at the 3-carbon atom is more stringent than for the peptide backbone determined by the stereochemistry of the 2-carbon atom. This can be most satisfactorily visualised as involving a three-point attachment for the terminal methyl-carbinol CH<sub>3</sub>CH(OH) group, involving the methyl and hydroxyl group and consisting of strict specificity for the relative position of the CH<sub>3</sub>, OH and H substituents with respect to one another, together with linkage to the peptide backbone. The relative position of the methyl-carbinol group with respect to the peptide backbone is less important than its absolute configuration.

The reaction mechanisms that have been considered to date [6–9] have all been concerned predominantly with the conformation of the acceptor peptide in free solution, whereas the data reported here would seem to indicate that much greater attention needs to be paid to possible interactions between the binding site of the OST protein and the acceptor peptide. Neither of the main models that have been proposed, in which the threonine hydroxyl group is postulated as interacting with the asparagine side-chain, satisfy a priori the requirement for strict stereospecificity as far as the 3-carbon atom is concerned, since neither model takes into account an interaction of the acceptor peptide with the methyl group.

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### References

- [1] Kornfeld, R. and Kronfeld, S. (1978) *Annu. Rev. Biochem.* 54, 631–664.
- [2] Marshall, R.D. (1972) *Annu. Rev. Biochem.* 41, 673–702.
- [3] Bause, E. (1979) *FEBS Lett.* 103, 296–299.
- [4] Welpy, J.K., Shenbagamurthi, P., Lennarz, W.J. and Naidner, F. (1983) *J. Biol. Chem.* 258, 11856–11863.
- [5] Lehle, L. and Bause, E. (1984) *Biochim. Biophys. Acta* 799, 246–251.
- [6] Bause, E. and Legler, G. (1981) *Biochem. J.* 195, 639–644.

- [7] Imperiali, B., Shannon, K.L., Unno, M. and Rickett, K.W. (1992) *J. Am. Chem. Soc.* 114, 7944–7945.
- [8] Imperiali, B., Spencer, J.R. and Struthers, M.D. (1994) *J. Am. Chem. Soc.* 116, 8424–8425.
- [9] Bause, E., Breuer, W. and Peters, S. (1995) *Biochem. J.* 312, 979–985.
- [10] Bause, E. (1983) *Biochem. J.* 209, 331–336.
- [11] Bause, E. and Hettkamp, H. (1979) *FEBS Lett.* 108, 341–344.
- [12] Bodanszky, M. and Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin.
- [13] Klein, R.A. (1979) *J. Chromatogr.* 170, 468–472.
- [14] Chenault, H.K., Dahmer, J. and Whitesides, G.M. (1989) *J. Am. Chem. Soc.* 111, 6354–6364.
- [15] Bause, E., Hettkamp, H. and Legler, G. (1982) *Biochem. J.* 195, 639–644.
- [16] Bray, G.A. (1970) *Anal. Biochem.* 1, 279–285.
- [17] Hart, G.W., Brew, K., Grant, G.A., Bradshaw, R.A. and Lennarz, W.J. (1979) *J. Biol. Chem.* 254, 9747–9753.