Epoxyethylglycyl peptides as inhibitors of oligosaccharyltransferase: double-labelling of the active site*

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Pig liver oligosaccharyltransferase (OST) is inactivated irreversibly by a hexapeptide in which threonine has been substituted by epoxyethylglycine in the Asn-Xaa-Thr glycosylation triplet. Incubation of the enzyme in the presence of Dol-*PP*-linked [^{14}C]oligosaccharides and the *N*-3,5-dinitrobenzoylated epoxy derivative leads to the double-labelling of two subunits (48 and 66 kDa) of the oligomeric OST complex, both of which are involved in the catalytic activity. Labelling of both subunits was blocked competitively by the acceptor peptide *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ and by the OST inhibitor *N*-benzoyl-α,γ-diaminobutyric acid-Gly-Thr-$NHCH_3$, but not by an analogue derived from the epoxy-inhibitor by replacing asparagine with glutamine. Our data clearly show that double-labelling is an active-site-directed modification, involving inhibitor glycosylation at asparagine and covalent attachment of the glycosylated inhibitor, via the epoxy group, to the enzyme. Double-labelling of OST can occur as the result of either a consecutive or a *syn*-catalytic reaction sequence. The latter mechanism, during the course of which OST catalyses its own 'suicide' inactivation, is more likely, as suggested by indirect experimental evidence. The *syn*-catalytic mechanism corresponds with our current view of the functional role of the acceptor site Thr/Ser acting as a hydrogen-bond acceptor, not a donor, during transglycosylation [Bause, Breuer and Peters (1995) Biochem. J. **312**, 979–985].

INTRODUCTION

Oligosaccharyltransferase (OST; EC 2.4.1.119), an integral component of the endoplasmic reticulum membrane, occupies a central position in the pathway of N-glycoprotein biosynthesis, because the enzyme links the dolichyl phosphate (Dol-*P*)-mediated branch of glycolipid formation with the Dol-*P*-independent route of oligosaccharide processing [1]. The enzyme has been purified from dog pancreas, pig and human liver, chicken oviduct and yeast [2–7]. In all cases the catalytic activity was found to be associated with an oligomeric membrane complex which, although differing between species in the overall number of subunits, always contains a 48 kDa protein as well as ribophorin I and II. Clear-cut evidence for specific functions of the individual subunits is still lacking. It may be, however, that the 48 kDa subunit is responsible for catalytic activity.

OST catalyses, in a co-translational event, the *en bloc* transfer of dolichyl diphosphate (Dol-*PP*)-activated oligosaccharides on to specific asparagine residues, provided that these are part of an Asn-Xaa-Thr/Ser signal sequence [8,9]. Model studies of this triplet sequence have shown that the hydroxyamino acid is not only important for substrate recognition and binding but also participates actively, via hydrogen-bond interactions, in transglycosylation. Two mechanistic models describing this catalytic function are currently under discussion [10–12]. The catalytic model put forward by Imperiali et al. [10] postulates that hydrogen-bond contacts are formed between the β-carbonyl group of the acceptor asparagine and the α-NH and β-OH groups of the hydroxyamino acid. These interactions, which are favoured by an Asx-turn conformation, are postulated to increase the asparagine β-amide acidity, so that a proton is dissociated. The resulting imidate structure then acts as a competent nucleophile in glycosylation. The alternative catalytic model, which we favour [11,12], proposes that the β-amide nucleophilicity of asparagine is enhanced by an 'inverted' hydrogen-bond interaction between the β-amide NH_2 protons as donor and the β-OH group oxygen of the hydroxyamino acid as acceptor. In the course of glycosylation the N–H···O bonded hydrogen is transferred to the hydroxy amino acid, which simultaneously delivers its own β-OH proton to a base at the active site. Based on this 'proton-vehicle' function of the hydroxyamino acid, we have previously designed a peptide derivative containing epoxyethylglycine in the hydroxyamino acid position of the Asn-Xaa-Thr/Ser motif [13]. This epoxy-derivative inactivated OST activity in calf liver crude microsomes in a time- and concentration dependent manner. The inhibitory effect was reduced markedly, however, when the endogenous pool of Dol-*PP*-linked oligosaccharide precursors was depleted prior to inhibitor addition. We concluded from these observations that OST catalyses its own inactivation. During this process the epoxy-inhibitor is thought to be glycosylated at asparagine and linked simultaneously, via the epoxy function, to a base at the active site.

The objective of the present work was to clarify this suicide inactivation by identifying the OST subunit(s) that may be linked covalently to the glycosylated epoxy-inhibitor. We show that two polypeptides of the oligomeric OST complex are double-labelled specifically on incubation of the pig liver enzyme with Dol-*PP*-[^{14}C]oligosaccharides and an *N*-dinitrobenzoylated epoxy-inhibitor peptide, detectable by immunological means. The molecular masses of these polypeptides (48 kDa and 66 kDa) are identical with those of two catalytically active subunits [3]. Subunit double-labelling was found to be blocked competitively by peptide derivatives that can act as glycosyl acceptors, whereas an analogue of the epoxy-inhibitor, in which asparagine was replaced with glutamine, was not effective. Our data support the suicide inactivation of OST by the epoxy-derivative and thus, indirectly, our view of the functional role of the hydroxyamino acid in Asn-Xaa-Thr/Ser during catalysis [11,12].

Abbreviations used: OST, oligosaccharyltransferase; Dol-*P*, dolichyl phosphate; Dol-*PP*, dolichyl diphosphate; Dnp-, dinitrophenyl-; Dnb-, dinitrobenzoyl-; -NHS, -*N*-hydroxysuccinimide; Boc-, t-butoxycarbonyl-; Vgly, vinylglycine; IIDQ, 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline.

* Dedicated to Professor Günter Legler on the occasion of his 70th birthday.

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MATERIALS AND METHODS

Materials

Materials and chemicals were obtained from the following sources: GDP-[^{14}C]mannose (specific radioactivity 303 mCi/mmol) and UDP-*N*-acetyl-[^{14}C]glucosamine (specific radioactivity 300 mCi/mmol) from Amersham; di-t-butylpyrocarbonate, benzylchloroformate, 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (IIDQ), *N*,*N*′-dicyclohexylcarbodi-imide, benzoylchloride, 3,5-dinitrobenzoylchloride and Triton X-100 from Fluka; trifluoroacetic acid, *m*-chloroperbenzoic acid and silica-gel G 60 plates from Merck; *N*-Boc-L-amino acids (where Boc- is t-butoxycarbonyl-) from Serva; Dol-*P*, biotinylated alkaline phosphatase and streptavidin from Sigma; tri-Boc-L-arginine and valine t-butylester hydrochloride from Bachem; 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium chloride from Boehringer; rabbit anti-2,4-dinitrophenyl (anti-Dnp) antibody and biotinylated goat anti-(rabbit IgG) antibody from Dako. All other chemicals used were of analytical grade.

Peptide synthesis

The various peptide derivatives were synthesized in solution by adapting published procedures [14,15]. Their purity was checked by TLC on Silica gel G 60 using n-butanol/acetic acid/water (4:1:1, by vol.), chloroform/methanol/acetic acid (65:25:5, by vol.) and propan-1-ol/aq. 34 % (w/v) NH_3 (7:3, by vol.). Their structures were confirmed by ^{1}H-NMR and MS.

N-Dinitrobenzoyl (Dnb)-Arg-Asn-Ala-epoxyethylglycine-Ala-Val (**I**) and *N*-benzoyl-Arg-Gln-Ala-epoxyethylglycine-Ala-Val (**II**).

The epoxy-derivative (**I**) and its glutamine analogue (**II**) were synthesized by coupling the C-terminal Vgly-Ala-Val fragment (where Vgly is vinylglycine) with *N*-Boc-Arg(Boc)$_2$-Asn-Ala-NHS and *N*-Boc-Arg(Boc)$_2$-Gln-Ala-NHS respectively (where NHS is *N*-hydroxysuccinimide) in 50 % dimethyl formamide in water, pH 8.5. After removal of the Boc groups with trifluoroacetic acid, the free hexapeptides were purified by chromatography on a silica gel RP 18 column (27 cm × 2.5 cm), using a methanol/water gradient. This was followed by *N*-(dinitro)-benzoylation with (dinitro)benzoyl-chloride in methanol/triethylamine. The Vgly CH_2=CH_2 group was converted into the epoxide by treatment with a 10-fold molar excess of *m*-chloroperbenzoic acid in acetic acid [13]. The epoxy-derivatives were precipitated with ether and the precipitates were extracted several-fold with ether to remove residual *m*-chloro(per)benzoic acid. The epoxy peptides were then dissolved in water and, after adjusting the pH to 7.3, used directly in the inactivation assay.

MS data were as follows: Arg-Asn-Ala-Vgly-Ala-Val, *m*/*z* 613 (*M*H$^+$); Dnb derivative, *m*/*z* 807 (*M*H$^+$); epoxy derivative **I**, *m*/*z* 823 (*M*H$^+$); Arg-Gln-Ala-Vgly-Ala-Val, *m*/*z* 627 (*M*H$^+$); *N*-benzoyl-Arg-Gln-Ala-Vgly-Ala-Val, *m*/*z* 731 (*M*H$^+$); epoxy-derivative **II**, *m*/*z* 747 (*M*H$^+$).

N-Boc-Arg(Boc)$_2$-Asn/Gln-Ala

N-Boc-asparagine- or *N*-Boc-glutamine-4-nitrophenyl ester was dissolved in dimethyl formamide and equivalent amounts of alanine-*O*-benzyl ester/tosylate and triethylamine were added. The mixture was kept at room temperature for 24 h and diluted with 10 vol. of ethyl acetate. The organic phase containing the *N*-Boc-Asn/Gln-Ala-*O*-benzyl ester was extracted with 10 % $NaHCO_3$/water, 5 % citric acid/water and then water, followed by drying over Na_2SO_4. After evaporation of the solvent, the solid residue was crystallized from hot ethyl acetate and the Boc group was cleaved by treatment with trifluoroacetic acid. *N*-Boc-Arg(Boc)$_2$ was dissolved in dimethyl formamide and its α-carboxy group activated by addition of 1.1 molar equivalents of dicyclohexylcarbodi-imide and 2 molar equivalents of NHS. After 10 h at 4 °C, an equivalent amount of Asn-Ala-*O*-benzyl ester or Gln-Ala-*O*-benzyl ester was added. The pH was adjusted to 8–9 with triethylamine and the reaction mixtures were kept at room temperature for 24 h. Precipitated dicyclohexylurea was removed by filtration, followed by dilution of the filtrates with 10 vol. of ethyl acetate. The organic phases were extracted and processed as described above. The oily residues obtained after evaporation of the solvent were chromatographed on a silica gel column (29 cm × 3 cm) using a stepwise gradient of ethyl acetate/acetone for elution. Cleavage of the benzyl ester group by hydrogenation over palladium/carbon yielded homogeneous preparations of the *N*-Boc-Arg(Boc)$_2$-Asn/Gln-Ala tripeptides.

MS and NMR data were as follows. *N*-Boc-Arg(Boc)$_2$-Asn-Ala-*O*-benzyl ester: *m*/*z* 750 (*M*H$^+$); ^{1}H-NMR δ 1.25 (CH_3-Ala), 1.3–1.5 (Boc$_3$-Arg, β-CH_2-Arg), 1.6 (γ-CH_2-Arg), 2.4 (β-CH_2-Asn), 3.75 (δ-CH_2-Arg), 3.9 (α-CH-Arg), 4.25 (α-CH-Ala), 4.5 (α-CH-Asn), 5.1 (CH_2-Phe), 6.8–7.4 (C_6H_5). *N*-Boc-Arg(Boc)$_2$-Asn-Ala, *m*/*z* 660 (*M*H$^+$); *N*-Boc-Arg(Boc)$_2$-Gln-Ala-*O*-benzyl ester, *m*/*z* 764 (*M*H$^+$); *N*-Boc-Arg(Boc)$_2$-Gln-Ala, *m*/*z* 674 (*M*H$^+$).

Vgly-Ala-Val

Equivalent molar amounts of Val-*O*-t-butyl ester and *N*-carbobenzoxy-Ala-NHS were dissolved in dimethyl formamide and 1.1 molar equivalents of triethylamine was added. After 24 h at room temperature, the incubation mixture was diluted with 10 vol. of ethyl acetate and the organic phase extracted with 10 % $NaHCO_3$/water, 5 % citric acid/water and water, followed by drying over Na_2SO_4. After evaporation of the solvent, the blocked dipeptide was taken up in propan-2-ol and the carbobenzoxy group was cleaved by catalytic hydrogenation over palladium/carbon. The resulting Ala-Val-*O*-t-butyl ester derivative was reacted with Boc-Vgly in methanol using IIDQ for activation, followed by removal of the *N*-Boc and *O*-t-butyl ester groups with trifluoroacetic acid. The unblocked Vgly-Ala-Val tripeptide was purified by silica gel RP 18 chromatography (column size 27 cm × 2.5 cm) using a water/methanol gradient for elution.

MS and NMR data were as follows. *N*-Boc-Vgly-Ala-Val-*O*-t-butyl ester: *m*/*z* 428 (*M*H$^+$); ^{1}H-NMR δ 0.85 (β-CH_3-Val), 1.25 (CH_3-Ala), 1.45 (Boc), 2.0 (β-CH-Val), 4.9 (α-CH-Val), 4.4 (α-CH-Ala), 4.6 (α-CH-Vgly), 5.1–5.3 (γ-CH_2-Vgly), 5.7 (β-CH-Vgly). Vgly-Ala-Val: *m*/*z* 272 (*M*H$^+$); ^{1}H-NMR δ 0.7 (β-CH_3-Val), 1.2 (CH_3-Ala), 1.9 (β-CH-Val), 4.05–4.10 (α-CH-Val/α-CH-Vgly), 4.3 (α-CH-Ala), 5.20–5.35 (γ-CH_2-Vgly), 5.9 (β-CH-Vgly).

OST assays

Standard incubation mixtures for measuring OST activity contained 50 mM Tris/HCl, pH 7.2, 10 mM $MnCl_2$, 0.8 % Triton X-100, 0.3 % phosphatidylcholine, 3000 c.p.m. of Dol-*PP*-[^{14}C]GlcNAc$_2$ and 1 mM acceptor tripeptide (*N*-benzoyl-Asn-Gly-Thr-$NHCH_3$) in 100 μl. Reactions were started by the addition of microsomes or aliquots of the partially purified OST preparation. After 15 min at 25 °C, incubations were stopped by the addition of 0.5 ml of methanol. [^{14}C]Glycopeptides were then isolated and quantified as described previously [12]. One unit of OST activity is defined as the transfer of 1 % [^{14}C]GlcNAc$_2$ from

Dol-*PP* to the acceptor peptide/min under our standard assay conditions. The presence of endogenous glycolipids in the OST preparations was determined by incubating pig liver crude microsomes, or the partially purified enzyme, with [^{3}H]acetyl-Asn-Gly-Thr-$NHCH_3$. Glycosylated and non-glycosylated ^{3}H-labelled peptides were separated by paper chromatography using propan-2-ol/acetic acid/water (29:4:9, by vol.).

Inhibition studies with pig liver crude microsomes

The effect of epoxy-derivatives **I** and **II** on the OST activity in crude microsomes was measured by preincubation at 25 °C in 100 μl of 50 mM Tris/HCl, pH 7.5, 0.5% Triton X-100, 5 mM $MnCl_2$, epoxy-derivative and microsomes. At given times the residual enzyme activity was assayed using 3000 c.p.m. of Dol-*PP*-[^{14}C]$GlcNAc_2$ and 1 mM *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$.

Double-labelling experiments

A solution of Dol-*PP*-[^{14}C]oligosaccharides (10^5 c.p.m.) in chloroform/methanol/water (10:10:3, by vol.) was dried under vacuum. The residue was taken up in 400 μl of buffer (see standard assay) containing approx. 200 units of partially purified OST, and the mixture was incubated with various concentrations of epoxy-inhibitor **I**. After 30 min at 25 °C, the reaction was stopped by the addition of methanol and the protein fraction was precipitated using the Wessel and Flügge procedure [16]. The protein pellet was dissolved in sample buffer and subjected to SDS/PAGE under reducing conditions, followed by electrophoretic transfer of the proteins on to nitrocellulose. Polypeptides containing covalently bound 3,5-dinitrobenzoylated inhibitor peptide were identified using a specific anti-Dnp antibody as the probe. Incorporation of ^{14}C was determined by TLC radioscanning. Controls were run under similar conditions either in the absence of inhibitor peptide **I** or in the presence of the glutamine-substituted epoxy-derivative **II**.

General methods

The following procedures were carried out as described in the literature: synthesis of Vgly [13]; synthesis of *N*-benzoyl-α,γ-diaminobutyric acid-Gly-Thr-$NHCH_3$ and *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ [12]; OST purification [3]; SDS/PAGE [17]; Western blotting and immunoblotting [18,19]; synthesis of Dol-*PP*-[^{14}C]$GlcNAc_2$ and Dol-*PP*-[^{14}C]oligosaccharides [20,21]; acid glycolipid hydrolysis and NaB^3H_4 reduction [22]. Radioactivity was determined by scintillation counting using Bray's solution [23]. Radioactively labelled bands on Western/immunoblots were detected and quantified with an automatic TLC Linear Analyser (Multi-Tracemaster 20; Berthold). The molecular mass (MH^+) of peptides was determined on a VG ZAB FAB mass spectrometer. ^{1}H-NMR data were recorded in [^{2}H]DMSO/^2H_2O using a Bruker AMX 500 MHz spectrometer.

RESULTS AND DISCUSSION

Design and synthesis of epoxy-peptides as inhibitors of OST

Previous studies with calf liver crude microsomes suggested that the inactivation of OST by Arg-Asn-Ala-epoxyethylglycine-Ala-Val-OCH_3 takes place by a mechanism involving self-destruction [13]. As part of this suicide mechanism, the inhibitor was assumed to be glycosylated and covalently linked to the active site of the enzyme. In order to investigate further this novel reaction sequence, the following experimental strategy was applied. The epoxy-substituted inhibitor peptide was labelled with the hapten group Dnb to allow its detection by immunological techniques, the glycolipid substrates involved in the inactivation reaction were radiolabelled with D-[U-^{14}C]mannose, and finally the incorporation of both Dnb and the ^{14}C label into OST was interpreted to indicate that the suicide mechanism was operative.

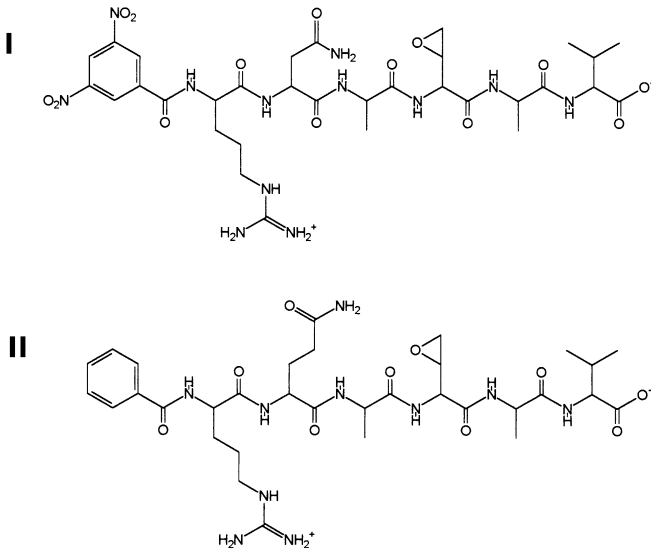

Figure 1 Structure of the epoxy-inhibitor (I) and its glutamine analogue (II)

The structure of the epoxy-substituted inhibitor peptide **I**, which we used in the double-labelling experiments, and the structure of the inhibitor analogue **II**, derived from **I** by replacing asparagine with glutamine, are shown in Figure 1. The amino acid sequence of **I** is identical to the sequence of the epoxy-hexapeptide that we used previously for inactivation studies with calf liver microsomes [13]. Structural differences include, however: (i) the lack of a C-terminal methyl ester group (this increased the solubility of **I** in buffer), and (ii) the presence of a Dnb group at the N-terminus, which allowed the immunological identification of covalent reaction products. The *meta*-substituted Dnb group was chosen as the hapten, because 2,4-dinitrobenzoylated peptides turned out to be unstable during the epoxidation reaction and the 3,5-substituted compounds were also recognized by the specific anti-Dnp antibody.

The epoxy-derivatives **I** and **II** were synthesized in solution using adaptations of published procedures (for details, see the Materials and methods section). L-Amino acids were used throughout, with the exception of Vgly, which was a racemate. D/L-Vgly enantiomers were not separated because we observed racematizion under the conditions of peptide synthesis. Thus we give the concentrations of all epoxy-derivatives as one-quarter of their total concentration, tentatively assuming that the incorporation of D/L-Vgly and the epoxidation of the double bond, introducing a new chiral centre, occur with similar efficiency, and that only the diastereomeric species of **I** containing L-*threo*-epoxyethylglycine is inhibitory.

The lipid-linked [^{14}C]oligosaccharides were prepared using GDP-[^{14}C]mannose as donor [21]. A severe problem, which decreased the sensitivity of the double-labelling experiments markedly, was that GDP-[^{14}C]mannose of only moderate specific radioactivity was commercially available. Furthermore, the [^{14}C]glycolipid preparation synthesized in the presence of microsomes was found to be diluted more than 150-fold by endogenous glycolipid precursors. This was shown by weak acid hydrolysis of

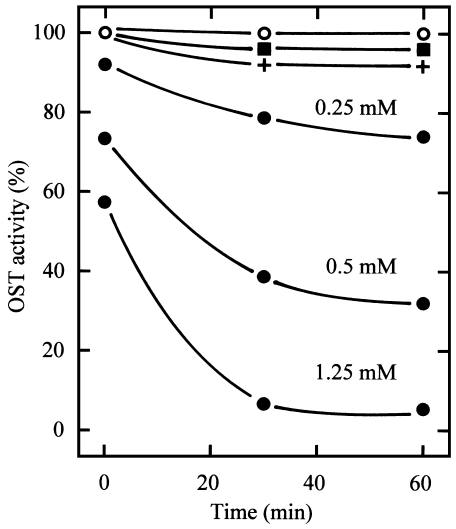

Figure 2 Time- and concentration-dependent inactivation of OST in pig liver crude microsomes

Microsomes were incubated in the presence of 0.5% Triton X-100 and various concentrations of Dnb-Arg-Asn-Ala-epoxyethylglycine-Ala-Val (**I**). At fixed times the residual OST activity was determined using Dol-*PP*-[^{14}C]GlcNAc$_2$ and *N*-benzoyl-Asn-Gly-Thr-NHCH$_3$ (see the Materials and methods section). ○, Control; ●, inhibitor **I**; +, epoxy-derivative **II** (1.25 mM); ■, epoxyethylglycine (2.5 mM).

a Dol-*PP*-[^{14}C]oligosaccharide fraction and reduction of the free [^{14}C]oligosaccharides with NaB^{3}H$_4$ of known specific radioactivity. These limitations could be avoided, at least partially, by using concentrated OST preparations.

Inactivation of OST in pig liver crude microsomes

The inhibitory potential of **I** was analysed by preincubating pig liver crude microsomes in the presence of various concentrations of the epoxy-derivative. At fixed times the residual OST activity was determined with Dol-*PP*-[^{14}C]GlcNAc$_2$ as donor and *N*-benzoyl-Asn-Gly-Thr-NHCH$_3$ as glycosyl acceptor. The results in Figure 2 show that the OST activity in the microsomal fraction was inactivated completely after approx. 30 min in the presence of 1.25 mM inhibitor **I**. The enzymic activity at zero time was found to differ depending on the inhibitor concentration in the assay. This is not surprising, as inactivation would already have taken place during the incubation time (15 min) needed for measuring residual OST activity. The time course and concentration-dependence of the inactivation of pig liver OST are comparable with those of the calf liver enzyme using Arg-Asn-Ala-epoxyethylglycine-Ala-Val-OCH$_3$ as inhibitor [13]. This indicates that the inhibitory potential of **I** is not affected either by the N-terminal Dnb group or by the lack of the C-terminal methyl ester group. Only marginal effects on enzymic activity were seen on incubating pig liver microsomes in the presence of either 2.5 mM epoxyethylglycine or 1.25 mM *N*-benzoyl-Arg-Gln-Ala-epoxyethylglycine-Ala-Val derivative **II** (Figure 2). Thus the inhibition by **I** is likely to be specific rather than non-specific, i.e. it is not caused by reactions such as denaturation.

Active-site double-labelling of pig liver OST

All double-labelling experiments were carried out using a concentrated OST preparation partially purified from pig liver microsomes as described in [3]. This was necessary for the following reasons. (i) The low amount of OST protein and the low specific radioactivity of the Dol-*PP*-[^{14}C]oligosaccharides made unambiguous identification of labelled reaction products in the crude microsomal fraction difficult. (ii) Glycosylation of the *N*-[^{3}H]acetyl-Asn-Gly-Thr-NHCH$_3$ acceptor peptide showed that crude microsomes contain considerable amounts of Dol-*PP*-linked donor oligosaccharides, whereas the [^{3}H]tripeptide was not glycosylated when incubated with the partially purified enzyme. Thus endogenous precursors compete in the inactivation reaction and decrease the extent of double-labelling. The presence of endogenous glycolipids is in line with the inactivation of OST when crude microsomes were incubated with the epoxy-inhibitor

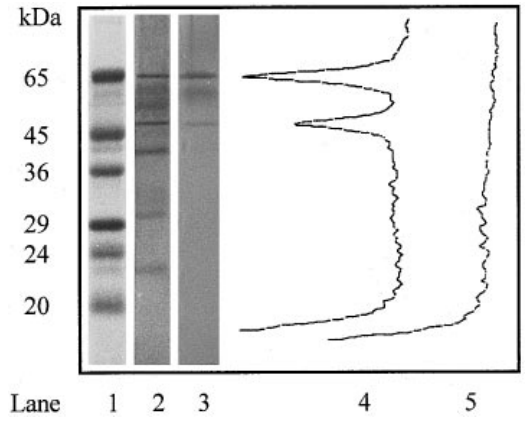

Figure 3 Active-site double-labelling of pig liver OST

A partially purified OST preparation containing ~ 200 enzyme units was incubated at 25 °C in the presence of 10^5 c.p.m. of Dol-*PP*-[^{14}C]oligosaccharides and 1.25 mM inhibitor **I**. After 30 min the reaction was stopped by the addition of methanol and the precipitated proteins were analysed by SDS/PAGE. Polypeptides that had acquired the ^{14}C and immunolabel were identified as described in the Materials and methods section. Lane 1, molecular mass standards; lane 2, Coomassie Blue stain; lane 3, immunoblot using an anti-Dnp antibody for detection; lane 4, ^{14}C incorporation into proteins of lane 2; lane 5, ^{14}C labelling in the absence of inhibitor **I**.

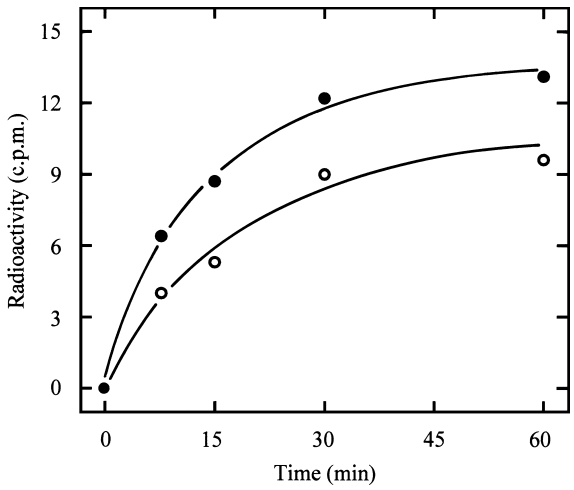

Figure 4 ^{14}C labelling of OST subunits as a function of time

OST (~ 200 enzyme units) was incubated at 25 °C in the presence of 10^5 c.p.m. of Dol-*PP*-[^{14}C]oligosaccharides and 1.25 mM epoxy-derivative **I**. At the given times the reactions were stopped by the addition of 0.5 ml of methanol and the samples were processed as described in the Materials and methods section. ●, 66 kDa subunit; ○, 48 kDa subunit.

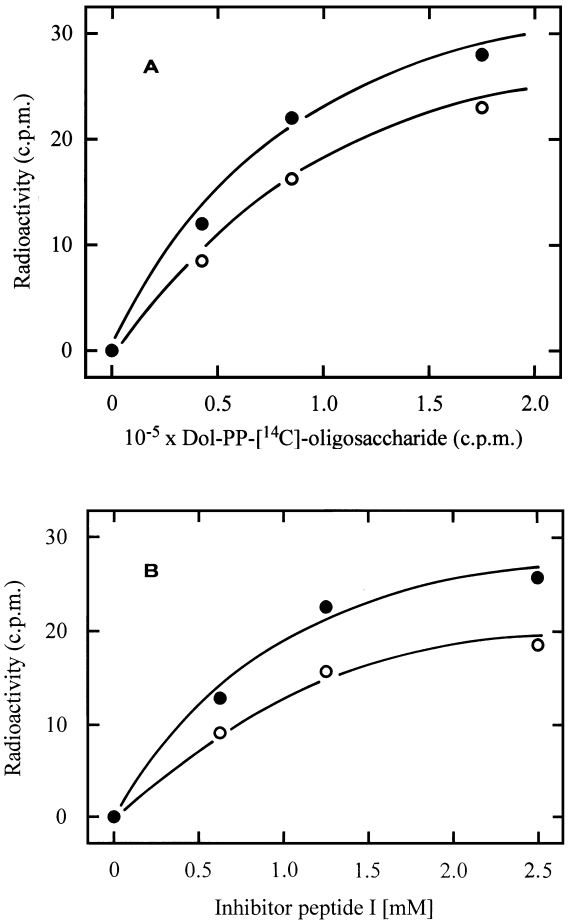

Figure 5 ^{14}C labelling of the 48 kDa and 66 kDa subunits as a function of inhibitor and glycolipid concentrations

The assay conditions were as in Figure 3, except that the incubations were carried out in the presence of various concentrations of either Dol-*PP*-[^{14}C]oligosaccharide (**A**) or inhibitor **I** (**B**). ●, 66 kDa subunit; ○, 48 kDa subunit.

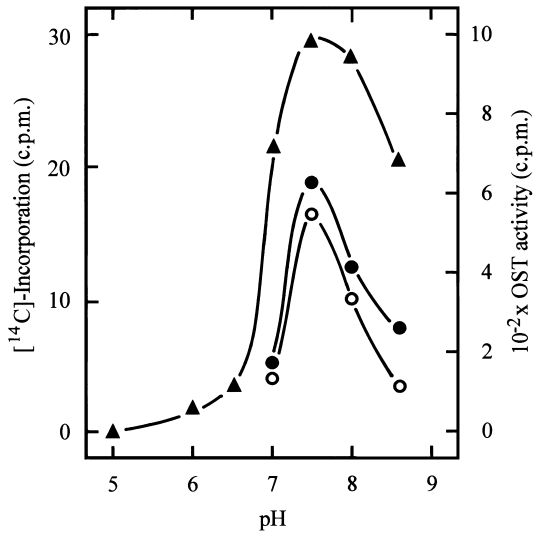

Figure 6 pH-dependence of OST activity and subunit ^{14}C labelling

OST activity (▲) was measured at various pH values in the presence of 2000 c.p.m. of Dol-*PP*-[^{14}C]GlcNAc$_2$ and 1 mM acceptor peptide *N*-benzoyl-Asn-Gly-Thr-NHCH$_3$. [^{14}C]Glycopeptide formation was then determined as described in [12]. The pH-dependence of ^{14}C incorporation was measured by incubating the enzyme in the presence of Dol-*PP*-[^{14}C]oligosaccharide (10^5 c.p.m.) and inhibitor peptide **I** (1.25 mM) for 30 min at a given pH. Radioactive proteins were quantified as described in the Materials and methods section: ●, 66 kDa subunit; ○, 48 kDa subunit.

I. (iii) The purified enzyme preparation contained several other polypeptides, in addition to the subunits of the oligomeric OST complex [3], and failure to double-label these was taken as an 'internal' control for the specificity of the reaction. (iv) Inhibitors **I** and **II** consisted of a synthetic mixture of diastereomeric peptides, and it is only the L-*threo* isomer that is likely to bind specifically to the enzyme. Since epoxides have a high alkylating potential, removal of the bulk of the contaminating proteins could be expected to minimize non-specific side reactions.

In a typical double-labelling experiment, OST (~ 200 enzyme units, as previously defined) was incubated in the presence of 10^5 c.p.m. of Dol-*PP*-[^{14}C]oligosaccharides and 1.25 mM derivative **I**. After 30 min the reaction was stopped by the addition of methanol. The proteins obtained after Wessel and Flügge precipitation [16] were separated by SDS/PAGE and transferred electrophoretically on to nitrocellulose. Inhibitor and [^{14}C]oligosaccharide incorporation was then assayed by radioscanning and immunostaining using an anti-Dnp antibody for detection (Figure 3). Coomassie Blue staining (lane 2) revealed the presence of several polypeptides in the OST preparation, including different subunits of the oligomeric OST complex (40, 48, 63 and 66 kDa). Of these polypeptides, only the 48 and 66 kDa species were both radioactively labelled (lane 4) and stained by the anti-Dnp antibody (lane 3). Thus they contain the inhibitor peptide and [^{14}C]oligosaccharides in covalent linkage. Incorporation of ^{14}C was not detectable when the enzyme was incubated with the Dol-*PP*-linked [^{14}C]oligosaccharides in the absence of inhibitor **I** (lane 5). This indicates that double-labelling requires the presence of both inhibitor and [^{14}C]glycolipids and, moreover, that ^{14}C incorporation is not due to glycosylation of putative Asn-Xaa-Thr/Ser sites in either protein.

We analysed the extent of [^{14}C]incorporation as a function of time, [^{14}C]glycolipid concentration and inhibitor concentration in order to characterize further the inactivation reaction. The results of these studies showed that ^{14}C labelling of the 48 kDa and 66 kDa polypeptides occurred with comparable efficiency and kinetics, increasing with time (Figure 4), [^{14}C]glycolipid concentration (Figure 5A) and inhibitor concentration (Figure 5B). In all experiments a somewhat lower (10–20 %) labelling rate of the 48 kDa protein was observed which was reproducible. Nevertheless the kinetic data are consistent with the time- and concentration-dependent inactivation of OST in crude microsomes (Figure 2). Calculation of the relative amount of ^{14}C incorporation, based on the specific radioactivity of the [^{14}C]glycolipids and the protein concentration estimated from the Coomassie Blue staining intensities (Figure 3, lane 2), indicated that approx. 15–25 % of the 48 kDa and 66 kDa polypeptides had acquired the ^{14}C label. This rather moderate yield suggests that non-specific reactions are superimposed upon the enzyme-specific inactivation, resulting in the destruction of catalytic activity and thereby preventing a higher degree of labelling. This view is supported by the observation that the ^{14}C labelling kinetics (Figure 5) are obviously non-linear over the whole concentration range. On the other hand, the ratio of specific/non-specific reactions appeared to differ depending on whether crude microsomes or partially purified OST preparations

were used. Thus the activity of the microsomal enzyme was, in contrast with that of the partially purified enzyme, barely affected by either epoxyethylglycine or the glutamine-containing inhibitor analogue **II** (see Figure 2). This is consistent with previous observations which showed that the catalytic activity of OST is markedly more unstable after purification [3]. The double-labelling reaction, on the other hand, is itself enzyme-specific and tightly associated with catalytic activity. This conclusion is supported by the observation that the pH-dependence of ^{14}C incorporation is the same as the pH profile for *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ glycosylation (Figure 6).

OST double-labelling is an active-site-directed reaction

In order to demonstrate that the inactivation of OST is an active-site-specific process, we incubated the enzyme in the presence of 10^5 c.p.m. of [^{14}C]glycolipids and 1.25 mM inhibitor analogue **II**. Under these conditions neither the 48 kDa nor the 66 kDa polypeptide was found to be radioactively labelled, consistent with the failure of **II** to affect OST activity in crude microsomes (Figure 2) or to inhibit subunit double-labelling in the simultaneous presence of **I** (results not shown). Thus both the epoxy group and the acceptor asparagine are essential structural elements for inactivation. These results also indicate that the asparagine residue in **I** is glycosylated during inactivation.

In contrast with the Gln-substituted derivative **II**, ^{14}C labelling of the 48 kDa and 66 kDa species was blocked efficiently in the presence of *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ (results not shown). Since the inhibition by the acceptor tripeptide could simply be due to [^{14}C]glycolipid depletion, we examined the effect on subunit labelling displayed by the substrate analogue *N*-benzoyl-α,γ-diaminobutyric acid-Gly-Thr-$NHCH_3$. This tripeptide is a competitive inhibitor of OST ($K_i \sim 0.2$ mM), but displays no glycosyl acceptor properties [12]. The results in Figure 7 show that the α,γ-diaminobutyric acid derivative inhibits the incorporation of ^{14}C label into both the 48 kDa and 66 kDa protein species. The effect was concentration-dependent, with 50% inhibition at ~ 0.3 mM, similar to the K_i. Thus double-labelling is an active-site-directed modification, requiring the glycosylation sequence to be completed and involving [^{14}C]glycolipids as well as the Asn-Xaa-epoxyethylglycine sequence in **I**.

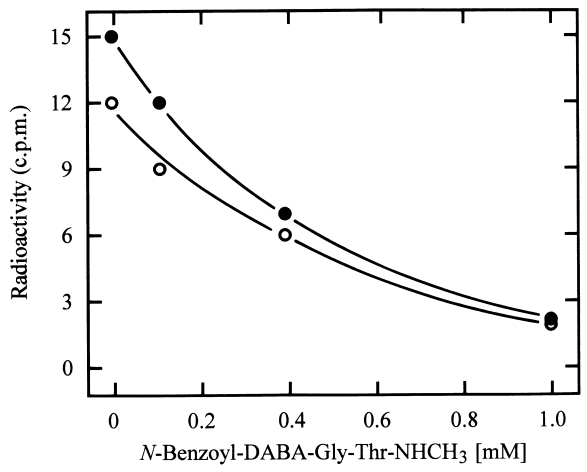

Figure 7 Effect of the competitive OST inhibitor *N*-benzoyl-α,γ-diaminobutyric acid-Gly-Thr-$NHCH_3$ on subunit labelling

OST (200 enzyme units) was incubated in the presence of Dol-*PP*-[^{14}C]oligosaccharide (10^5 c.p.m.), epoxy-inhibitor **I** (1.25 mM) and various concentrations of the α,γ-diaminobutyric acid (DABA)-containing tripeptide. After 30 min at 25 °C, the samples were processed as decribed in the Materials and methods section. ●, 66 kDa subunit; ○, 48 kDa subunit.

Conclusions

We describe here the irreversible inactivation of pig liver OST by a dinitrobenzoylated hexapeptide which contains epoxyethylglycine instead of Thr/Ser in the glycosylation triplet. Incubation of the enzyme with epoxy-derivative **I** and Dol-*PP*-[^{14}C]oligosaccharides gave rise to two doubly labelled polypeptides with molecular masses (48 kDa and 66 kDa) identical to those of subunits of the oligomeric OST complex. These subunits have been shown previously to be essential for catalytic activity [3]. Our data clearly imply that double-labelling is an active-site-directed modification during which the inhibitor is glycosylated at asparagine and the glycosylated peptide is covalently bound to the enzyme. An essential prerequisite for this active-site-directed inhibition is that the epoxy-inhibitor should be recognized by and specifically bound to the enzyme. This most probably occurs because the epoxy structure and the threonine side chain resemble one another sterically (Figures 8A and 8B). The epoxy-inhibitor used was, however, a mixture of diastereomeric peptides containing D/L-*threo*/*erythro* forms of epoxyethylglycine. We assume, in the face of no other evidence, that only the L-*threo* form, with a β-C-configuration identical to that of L-threonine, is the inhibitory species. This assumption is supported by previous studies which have shown that replacement of the L-threonine in the Asn-Xaa-Thr motif by L-*allo*-threonine impaired peptide recognition and glycosylation by OST [12].

Double-labelling of the OST subunits may occur by two different mechanisms: (i) a consecutive reaction sequence, or (ii) a *syn*-catalytic or suicide mechanism, during which the enzyme catalyses its own inactivation. The consecutive mechanism involves, after inhibitor binding, nucleophilic attack of appropriate base(s) on the γ-CH_2 group of the epoxy ring, resulting in covalent attachment of the inhibitor to the active site and simultaneous generation of an OH group at the β-C-atom. The formation of this β-OH group is obviously crucial for allowing the subsequent glycosylation of asparagine to take place [11,12]. This also follows from the observation that the epoxy-inhibitor itself cannot act as a glycosyl acceptor, in contrast to the diol obtained on hydrolysis of the oxiran ring (results not shown). The consecutive mechanism of inactivation would mean that the nucleophile(s) reacting with the oxiran ring most probably differ from those directly involved in OST catalysis.

The *syn*-catalytic mechanism, which we favour, assumes that the epoxide oxygen functions in a similar fashion to the β-OH group of threonine, namely as a hydrogen-bond acceptor that interacts with the asparagine $CONH_2$ group, thus enhancing the β-amide nucleophilicity (Figures 8A and 8B). Upon initiation of the glycosylation sequence, the β-amide hydrogen of asparagine is transferred to the oxiran ring oxygen, yielding an intermediate with high alkylating potential. In contrast with the β-hydroxy group of threonine, however, the protonated oxiran ring is presumed to alkylate the active-site base that would normally accept the β-hydroxy proton. Indirect evidence supporting this *syn*-catalytic rather than the consecutive mechanism is provided by previous studies which have shown that the inactivation of OST in calf liver crude microsomes was less pronounced when the endogenous pool of Dol-*PP*-linked oligosaccharides was depleted prior to addition of the epoxy-inhibitor [13]. This indicates that Dol-*PP*-oligosaccharides play an essential active role in the inactivation reaction. A similar effect of the glycolipid donor can be inferred from the observation that, on incubation

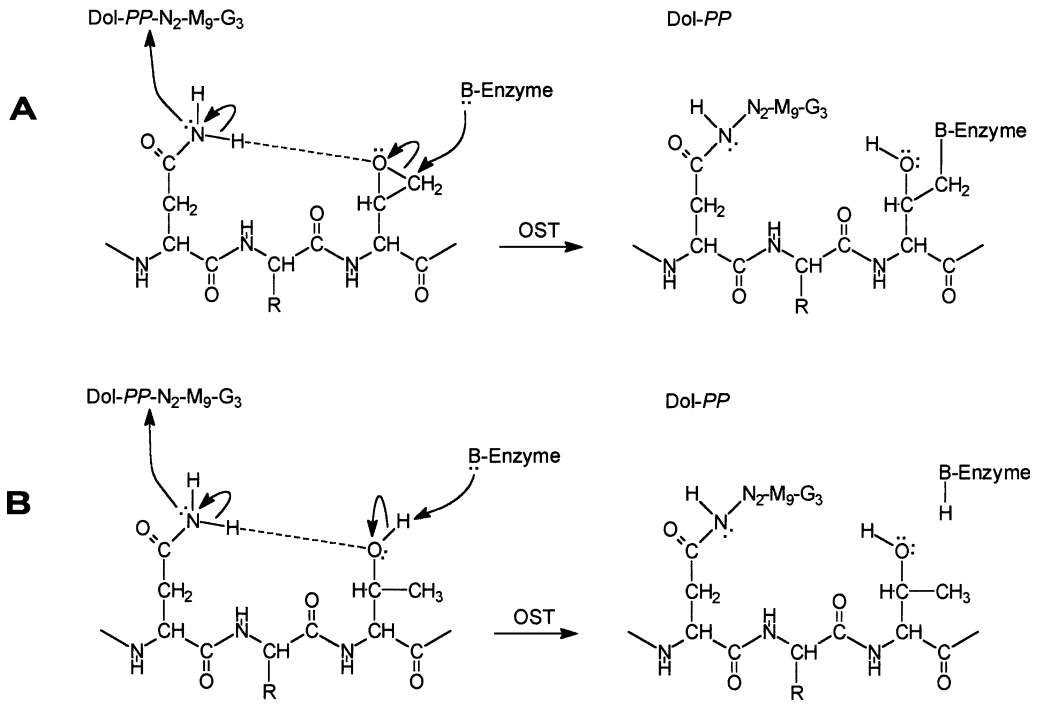

Figure 8 Models describing (A) the suicide inactivation of OST leading to subunit double-labelling, and (B) the catalytic mechanism of OST according to Bause et al. [11,12]

N, *N*-acetylglucosamine; M, mannose; G, glucose. See the text for details.

of crude microsomes with the epoxy-inhibitor, the rate of OST inactivation differed markedly depending on the assay system used for measuring residual enzyme activity (results not shown). Thus we found that the time-dependent loss of OST activity was much more rapid when determined with [^{3}H]acetyl-Asn-Gly-Thr-$NHCH_3$ as acceptor and endogenous Dol-*PP*-oligosaccharides as donor than with our standard assay (see the Materials and methods section). This apparent discrepancy in the inactivation rate is best explained by the assumption that the pool of endogenous glycolipids gradually declines during preincubation. This decline could result from reaction with the epoxy-derivative, after hydrolytic ring opening. The glycolipids are, therefore, available neither for [^{3}H]peptide glycosylation, thus leading to a higher apparent inactivation rate, nor for the inactivation reaction to proceed to completion. Although preliminary, these data support the view that double-labelling may indeed occur by the proposed suicide mechanism (Figure 8A). This would be in line with the catalytic mechanism of OST (Figure 8B) and, in particular, with the postulated role of the hydroxyamino acid in Asn-Xaa-Thr/Ser to act as a hydrogen-bond acceptor rather than a donor [11,12]. By contrast, the *syn*-catalytic mechanism of inactivation is obviously inconsistent with the catalytic model of transglycosylation proposed by Imperiali et al. [10], in which these authors postulate a hydrogen-bond donor function for the hydroxyamino acid. The consecutive mechanism of subunit double-labelling, on the other hand, would not contradict either catalytic model. Further studies still need to be carried out to distinguish between these two possibilities.

The reason why the two subunits (48 kDa and 66 kDa) of the OST complex became double-labelled is not clear. The dissociation of the tetrameric OST complex by concanavalin A–Sepharose chromatography gave a catalytically active fraction consisting of just these two subunits [3]. It appears likely, therefore, that both the 48 kDa and 66 kDa polypeptides display OST activity. Their apparently tight association with catalytic activity shows, at least, that the two subunits must be closely related within the membrane complex, in terms of both space and function. The 66 kDa polypeptide, representing ribophorin I, contains a highly hydrophobic domain, assumed to function as a potential binding site for Dol-*PP*-oligosaccharides [24]. Thus it may be possible that glycolipid and peptide binding, as well as glycosylation, require the concerted action of distinct peptide domains within the 48 kDa and 66 kDa subunits. Double-labelling of both polypeptides could occur when appropriate basic groups on either protein, which may or may not be involved in the catalytic mechanism, are modified in a random manner due to their close proximity to the (protonated) oxiran ring. This, at least, would explain why the relative ratio of the labelled products for the 48 kDa and 66 kDa subunits, although generated on a similar time scale, was found to vary between 0.7 and 0.9. We are currently trying to identify the [^{14}C]glycopeptide attachment sites in both polypeptides to address this question further.

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