The Ost1p Subunit of Yeast Oligosaccharyl Transferase Recognizes the Peptide Glycosylation Site Sequence, -Asn-X-Ser/Thr-\(^*\)

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Other laboratories have established that oligosaccharyl transferase (OST) from *Saccharomyces cerevisiae* can be purified as a protein complex containing eight different subunits. To identify the OST subunit that recognizes the peptide sites that can be glycosylated, we developed photoaffinity probes containing a photoreactive benzophenone derivative, \(p\)-benzoylphenylalanine (Bpa), as part of an \(^{125}\)I-labeled peptide that could be expected to be glycosylated. We found that Asn-Bpa-Thr peptides served as substrates for OST and that photoaacivation of these probes in the presence of microsomes abolished the OST activity. Photoactivation of \(^{125}\)I-labeled Asn-Bpa-Thr in the presence of microsomes resulted in specific covalent labeling of a protein doublet of molecular mass 62 and 64 kDa. By carrying out the photoactivation of the probe using microsomes containing epitope-tagged Ost1p, we demonstrated that the \(^{125}\)I-labeled protein was Ost1p. Radiolabeling of this protein was dependent on irradiation at 350 nm. No labeling was detected using a probe containing Ala instead of Thr as the third amino acid residue. We conclude that Ost1p is the subunit of the OST complex that recognizes the peptide sites in the nascent chains that are destined to be glycosylated.

The key enzyme in the process of N-glycosylation of proteins, oligosaccharyl transferase (OST), catalyzes the transfer of a preassemble high-mannose oligosaccharide from a lipid-linked oligosaccharide donor (Dol-PP-GlcNAc\(_2\)Man\(_9\)Glc\(_3\)) onto asparagine acceptor sites on nascent polypeptide chains being translocated into the lumen of the rough ER (1, 2). The consensus sequence of the acceptor, -Asn-X-Thr/Ser-, where X is any amino acid other than Pro (3, 4), was confirmed using a variety of synthetic peptides as substrates for *in vitro* glycosylation in microsomes (5–8).

OST in *Saccharomyces cerevisiae* was initially purified as a complex consisting of six polypeptides (\(\alpha, \beta, \gamma, \delta, \epsilon, \zeta\) subunits) with enzymatic activity (9). Subsequently, two other groups (10, 11) have described a tetrameric OST complex that lacks the \(\epsilon\) and \(\zeta\) subunits. So far in yeast eight subunits have been cloned that may be components of this enzyme complex. Five of them are essential genes (OST1, OST2, WBP1, SWP1, and STT3). The components of yeast OST complex show significant sequence similarity to the components of the complex purified from higher eukaryotes (12).

Much work has been done to characterize the substrates of OST (13–16), but little is known about the function of the subunits of the enzyme complex. Following the observation that modification of a cysteine residue on the OST complex by methyl methanethiosulfonate caused time- and concentration-dependent inactivation of enzyme activity, a biotin-tagged form of this reagent was shown to inactivate the enzyme and to label Wbp1p. Based on the finding that the substrate, Dol-PP-GlcNAc\(_2\), protected the enzyme from inactivation, it was proposed that Wbp1p may contain a site for the binding of the lipid-linked oligosaccharide (11). Following up on an earlier observation (16) on inactivation of pig liver OST by a hexapeptide in which Thr was replaced by epoxyethylglycine in the -Asn-X-Thr- consensus sequence, Bause et al. (17) used a \(N\)-dinitrobenzoylated form of this hexapeptide with the objective being to identify an OST subunit(s) that might become covalently linked to the epoxy inhibitor. Following incubation of the pig liver OST with Dol-PP-\(^{14}\)C-oligosaccharides and the \(N\)-dinitrobenzoylated hexapeptide, two polypeptides, proposed to be ribophorin I and Ost48p, were found to be immunolabeled and radioactive.

Our approach has been to identify one or more subunits of the yeast OST complex that would be expected to be involved in recognition of -Asn-X-Thr/Ser- glycosylation sites in the growing polypeptide chain. To accomplish this, we took advantage of the fact that small glycosylatable peptides can mimic nascent chains, because they can enter the ER and become glycosylated (5, 6, 8). Based on this fact, we developed photoreactive glycosylatable peptides containing a benzophenone moiety. Such probes have several advantages: 1) they are chemically more stable than other photoreactive moieties such as diazo esters, aryl azides, and diazirines; 2) can be manipulated in ambient light and can be activated at 350 nm, conditions that minimize damage to proteins; and 3) react preferentially with normally unreactive C–H bonds, even in the presence of aqueous solvent and bulk nucleophiles. These three properties combine to allow for highly efficient covalent modifications of macromolecules, frequently with high site specificity (18, 19). We used photoprobes containing the -Asn-X-Thr- consensus sequence in which the X amino acid was \(p\)-benzoylphenylalanine (Bpa). Using this probe we were able to show that Ost1p is the subunit in the OST complex that recognizes the N-glycosylation site sequence.

**EXPERIMENTAL PROCEDURES**

*Strains—*W303-1a (MAT a ade2 can1 his3 leu2 trp1 ura3) was used as the parental strain to generate the Ost1p hemagglutinin (HA) and c-Myc epitopes integrated into the chromosome. Polymerase chain reaction was carried out using the ME-3 plasmid (which contains a triple HA tag and the HIS5 gene from *Schizosaccharomyces pombe*) as the

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§ The abbreviations used are: OST, oligosaccharyl transferase; bh, Bolton-Hunter; Bpa, benzoylphenylalanine; Am, amide; Ac, acetyl; ConA, concanavalin A; ER, endoplasmic reticulum; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; Dol, dolichol.
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**RESULTS**

**Asn-Bpa-Thr Serves as a Substrate for OST.—**To search for the peptide recognition subunit(s) of OST, \(^{125}\text{I}-\text{bh}-\text{Asn-Bpa-Thr-Am}\) and \(^{3}\text{H}\text{-Ac}-\text{Asn-Bpa-Thr-Am}\) were incubated with yeast cell lysate without photoactivation at 350 nm. Labeled glycopeptide formed from the labeled peptide was quantitated by binding of the former to ConA-agarose beads as described (24). The results shown in Fig. 2 indicate that formation of a \(^{3}\text{H}\text{-labeled glycopeptide that binds to ConA-agrose beads, sonication using a Branson sonicator at level 3 was performed on the unlabeled peptide, Ac-Asn-Phe-Thr-Am, which is also a substrate for OST.**
with the labeled probe and a large excess of a peptide, Ac-Asp(NHCH$_2$)$_2$-Leu-Thr-Am, which is known to be inactive as a substrate for OST (27), labeling of the 62/64-kDa band was unaffected (Fig. 4, lane 5). Furthermore, when the labeled acceptor photoprobe was replaced with another labeled photoprobe that is not a substrate for OST, $^{125}$I-bh-Asn-Bpa-Ala-Am, which is not an OST substrate, was tested as a photoprobe. Lane 7, microsomes were heated at 100 °C for 5 min before photoactivation. Lane 8, EDTA was added to the reaction mix before photoactivation.

**The Radiolabeled Protein Is Ost1p**—Based on the specificity of anti-HA in precipitating only the OST subunits (25) and the apparent molecular mass of the photolabeled protein we presumed that the subunit being labeled was Ost1p. It has been reported Ost1p migrates as a triplet of protein bands at 60/62/64 kDa due to different glycosylation states (26), although as shown in Fig. 4, lane 2, we detected only a doublet at 62 and 64 kDa. To provide more direct proof that the labeled protein was Ost1p, photoactivation was carried out using microsomes prepared from a strain bearing chromosome-integrated HA tag located at the COOH terminus of Ost1p. Immunoprecipitation with anti-HA, followed by SDS-PAGE, revealed two labeled protein bands migrating at 67 and 65 kDa (Fig. 4, lane 3). The HA tag had a mass about 3 kDa, which explains why the doublet of labeled Ost1p now had a slower mobility than the Ost1p doublet observed when the epitope was attached to

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Irradiation of Microsomes and Labeled Asn-Bpa-Thr Yields an $^{125}$I-Labeled Protein That Is Immunoprecipitated by Antibody to HA-tagged Ost3p—Having established that $^{125}$I-bh-Asn-Bpa-Thr-Am and [$^{3}$H]Ac-Asn-Bpa-Thr-Am are substrates of OST and that irradiation of the unlabeled photoprobe peptide inactivated OST, we investigated the target of its binding. It has been shown that incorporation of the HA epitope into the COOH terminus of Ost3p, followed by cell rupture and then immunoprecipitation under nondenaturing conditions, results in precipitation of all eight subunits of the OST complex (25). After confirming this observation, we carried out experiments using microsomes prepared from a yeast strain in which an HA-tagged allele of Ost3p was integrated into the chromosome. Photoactivation of these microsomes in the presence of $^{125}$I-bh-Asn-Bpa-Thr-Am was followed by solubilization, and then immunoprecipitation with Ost3-HAp, in order to detect any radiolabeled OST subunits. The results shown in Fig. 4, lane 2, established that irradiation of microsomes prepared from the yeast strain carrying Ost3-HAp in the presence of $^{125}$I-bh-Asn-Bpa-Thr-Am produced a doublet of radiolabeled bands of apparent molecular masses of 62 and 64 kDa (Fig. 4, lane 2). As shown in Fig. 4, lane 1, when the microsomes were incubated with $^{125}$I-bh-Asn-Bpa-Thr-Am, in the absence of irradiation, followed by immunoprecipitation and subsequent SDS-PAGE, no labeled protein bands were detected. Therefore, the radiolabeling of these proteins was irradiation-dependent. In other experiments (data not shown) we have found that reduction of the photoreactive ketone group in the Bpa abolished its ability to become covalently linked to protein. In addition, when the photoactivation was carried out using microsomes that had been inactivated by either heating, or by adding 4-fold molar excess of EDTA (to deplete the divalent cations known to be necessary for OST activity), radiolabeling of these two bands did not occur (lanes 7 and 8, respectively).

To further confirm that photoactivation led to specific labeling of a polypeptide, a competition experiment was performed using a labeled probe and either an unlabeled peptide that is a known substrate for OST or a peptide that has been shown to not be a substrate. As demonstrated in Fig. 4, lane 4, when microsomes were incubated with $^{125}$I-bh-Asn-Bpa-Thr-Am with the presence of a large excess of a competing acceptor peptide, Ac-Asn-Phe-Thr-Am, labeling of the 62/64-kDa doublet did not occur. However, when microsomes were incubated
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In an earlier effort to identify the peptide recognition element of OST by photoactivation of an acceptor, we utilized a glycosylatable peptide containing a photoreactive benzoylazido group attached to the ϵ-NH₂ group of Lys located in the X-position (22). We found that this probe did not label a membrane protein, but instead the lumenal protein, protein disulfide isomerase (PDI) (28). Subsequently we excluded the possibility that labeling of a membrane protein failed because of the high abundance of PDI; even when PDI was depleted from a sequence within a growing polypeptide chain, the extended luminal domain of Ost1p (427 amino acid residues) could have a sufficiently high degree of order to generate a very structured, specific binding site that can distinguish between Asn and Gln, and between Ser/Thr and Ala. Another aspect of interest is the relationship between this finding in yeast and work in mammalian systems, since Ost1p is homologous to ribophorin I. As mentioned above, Bause et al. (17) have proposed that ribophorin I and Ost48p are involved in recognition of Dol-PP-oligosaccharide, and the work of Pathak et al. (11) in yeast has implicated Wbp1p, the yeast homolog of Ost48p, in recognition of Dol-PP-oligosaccharide. Clearly a next step in extending our findings on the participation of Ost1p in recognition of the peptide binding site will be to use a bifunctional cross-linking probe to determine the possible interaction of Ost1p with other subunits of the yeast OST complex.

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