In the yeast, *Saccharomyces cerevisiae*, oligosaccharyl transferase (OT) is composed of nine different transmembrane proteins. Using a glycosylatable peptide containing a photoprobe, we previously found that only one essential subunit, Ost1p, was specifically labeled by the photoprobe and recently have shown that it does not contain the recognition domain for the glycosylatable sequence Asn-Xaa-Thr/Ser. In this study we utilized additional glycosylatable peptides containing two photoreactive groups and found that these were linked to Stt3p and Ost3p. Stt3p is the most conserved subunit in the OT complex, and therefore 21 block mutants in the luminal region were prepared. Of the 14 lethal mutant proteins only two, as well as one temperature-sensitive mutant protein, were incorporated into the OT complex. Here, using microsomes prepared from these three strains, the labeling of Ost1p was markedly decreased upon photoactivation with the Asn-Bpa-Thr photoprobe. Based on the block mutants single amino acid mutations were prepared and analyzed. From all of these results, we conclude that the sequence from residues 516 to 520, WWDYG in Stt3p, plays a central role in glycosylation process.

Oligosaccharyl transferase (OT) transfers preassembled oligosaccharide chains from a lipid-linked oligosaccharide donor (Dol-PP-GlcNacMαManGlc) onto asparagine residues specified by the Asn-Xaa-Thr/Ser sequence, where Xaa can be any amino acid except proline (1–6). Biochemical, molecular biological, and genetic studies have led to the identification of a remarkably large number of subunits for yeast OT (7–20). During the last decade, OT complexes have been purified from different sources, such as dog pancreas (21, 22), hen oviduct (23), human (24) and pig liver (25), and yeast (10, 26, 27).

To identify the subunit(s) of yeast OT that recognizes Asn-Xaa-Thr/Ser sites that can be glycosylated, earlier we developed a photoaffinity probe containing a photoreactive benzophenone derivative, p-benzoylphenylalanine (Bpa) (28). By using this 125I-labeled Bpa-containing tripeptide, we found that Ost1p was specifically labeled by photoactivation in yeast microsomes (29). However, subsequently we found that Ost1p is not the glycosylation site recognition/catalytic subunit. In this study, we used additional sets of peptides containing two photoreactive groups for photoactivation and found that the photoprobe became linked to Stt3p. Stt3p is conserved in eukaryotes and essential for vegetative growth of yeast cells. In fact, it is the most conserved subunit among the OT components. Human, murine, and *Drosophila* proteins, as well as a putative protein in *Caenorhabditis elegans*, are more than 50% identical in amino acid sequence (17). Stt3p contains a very hydrophobic N-terminal domain spanning the membrane 10–12 times and a hydrophilic, luminal domain at the C terminus (16, 17).

To further investigate function of Stt3p, we prepared 21 block mutants in the luminal domain within regions of high conservation. Furthermore, single amino acid mutations that were derived from the block mutants were also prepared. Based on studies with these and other mutants, we conclude that Stt3p is the OT subunit involved in peptide recognition and/or catalysis and that the sequence at residues 516–520, WWDYG, plays a key role in this process.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**W303-1a (MAT a ade2 can1 his3 leu2 trplura3) was used as the parental strain to generate the Stt3p hemagglutinin (HA) construct that is integrated into the chromosome. PCR was carried out using the ME-3 plasmid (which contains a triple HA tag and/or catalysis and that the sequence at residues 516–520, WWDYG, plays a key role in this process.**

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resulting PCR product was subcloned into pRS314, which was digested with SmaI and SacI to generate the plasmid pRS314-STT3HA.

Materials—Asn-Bpa-Thr-Am was custom-made by Quality Controlled Biochemicals (Hopkin, MA), and Bpa-Ala-Asn-Ala-Thr-Ala-Bpa-Am and Asn-Bpa-Thr-Ala-Bpa-Am were custom-made by Center for the Analysis and Synthesis of Macromolecules (Stony Brook, NY). In each case the N terminus was derivatized with $^{125}$I-labeled Bolton-Hunter (bh) reagent (3 Ci/μmol; ICN, Irvine, CA) as described previously (30), yielding $^{125}$I-bh-Asn-Bpa-Thr-Ala-Bpa-Am, $^{125}$I-bh-Asn-Bpa-Thr-Am, and $^{125}$I-bh-Asn-Bpa-Thr-Ala-Bpa-Am, respectively. [H]Acetic anhydride (7.3 Ci/μmol; Amersham Biosciences) was used to prepare [H]Ac-Asn-Bpa-Thr-Am (31). Yeast microsomes were prepared as described by Baker et al. (32).

PCR Mutagenesis—PCR mutagenesis was performed according to the manufacturer’s protocol (Stratagene, La Jolla, CA). For all of the group and single-residue mutations mentioned in this paper, pRS314-STT3HA was used as the template. Mutagenized plasmids were sequenced, and those with the expected sequence were transformed into QY700. The transformants were selected for Trp and Ura prototrophy and then further for 5-fluoro-orotic acid selection.

Conditions for Photocatalytic and Immunoprecipitation—Yeast spheroplasts or crude microsomes were used for photolysis as described previously (29). After irradiation, immunoprecipitation was performed as described by Karaoglu et al. (33), except that after the photocatalytic step the reaction was adjusted to 1.5% digitonin, 0.5 mM NaCl, 20 mM Tris-Cl, pH 7.4, 3.5 mM MgCl₂. The solution was centrifuged for 20 min at 55,000 × g in a TLA100.3 rotor (Beckman, Fullerton, CA), and the supernatant fraction was used for immunoprecipitation.

Spotting Assay for Growth—To determine the growth difference between yeast cells carrying stt3p mutants, the same amount of cells (5 × 10⁶) were collected after the strains had been grown to early log phase in Trp media at 25 °C. Then 10 μl of serial 1:10 dilutions of the cells were spotted on Trp plates and incubated at 25, 30, and 37 °C, respectively.

Oligosaccharyl Transferase Activity Assay—To determine whether in fact $^{125}$I-bh-Asn-Bpa-Ala-Asn-Thr-Ala-Bpa-Am and $^{125}$I-bh-Asn-Bpa-Thr-Ala-Bpa-Am are substrates for OT, yeast microsomes (50 μg of total protein/reaction) were used for the assay. The assay was performed as described (34). To determine the OT activity of the stt3p mutants, yeast spheroplasts were prepared from each mutant and [H]Ac-Asn-Bpa-Thr-Am was used as the substrate. For each reaction, 50 μl of spheroplasts (equivalent to 5 A₂₆₀ units of cells) and 0.5 μCi of peptide substrate were used. After incubation at 25 °C for 20 min, the entire reaction was added to concanavalin Aagarose beads. After allowing glycosylated peptides to bind at room temperature for 4 h, concanavalin A beads were washed twice with 50 mM Tris-Cl, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40. The amount of the radioactivity on the beads was counted.

Endoglycosidase H Digestion—For the immunoprecipitated samples, the proteins were eluted from protein G-agarose beads with 0.5% SDS and 1% β-mercaptoethanol and then further for 5-fluro-orotic acid selection.

Additional OT Subunits Can Be Photolabeled Using Probes Other than Asn-Bpa-Thr—Previously we used $^{125}$I-bh-Asn-Bpa-Thr-Am for photocatalysis. It is known that the OT does not have a specific requirement for the middle amino acid, as long as it is not proline, which would introduce a rigid kink in the conformation of the peptide (1, 2). Although Bpa is located between Asn and Thr, the benzoyl side chain could be oriented away from the enzyme active site. Because we found that a tripeptide with Bpa in the middle position did not lead to photocatalysis of the active site, we synthesized and tested peptides in which two Bpa residues were incorporated with the hope that the photoprobe could act as a bifunctional probe and thereby cross-link two different proteins (or two different parts of the same protein). This peptide was found to be a substrate for OT, although it was not as effective as the original tripeptide (data not shown).

In a second experiment we studied this peptide in terms of photolabeling of OT subunits upon incubation with microsomes. After photocatalysis, the microsomes were solubilized with digitonin, and the clarified supernatant was used for non-denaturing immunoprecipitation. Microsomes from a strain containing a chromosomally integrated HA epitope-tagged form of the OST3 gene were used to do photolabeling with this heptapeptide. The proteins precipitated with anti-HA antibodies were fractionated on SDS-PAGE and subjected to

### RESULTS AND DISCUSSION

### Table I

| Probe structure | Proteins radiolabeled |
|----------------|-----------------------|
| $^{125}$I-bh-Asn-Bpa-Thr-Am | Ost1p |
| $^{125}$I-bh-Asn-Ala-Thr-Ala-Bpa-Am | Stt3p, Ost3p |
| $^{125}$I-bh-Asn-Ala-Thr-Ala-Thr-Am | Ost1p, Stt3p, Ost3p |

**TABLE I** Differential labeling of OT subunits by three different photoprobes

**Fig. 1.** Photolabeling of OT subunits using bifunctional photoprobes. Microsomes were prepared from each epitope-tagged strain, and photocatalysis was carried out. Photodized microsomes were subjected to immunoprecipitation as described under “Experimental Procedures.” The samples were analyzed by SDS-PAGE, and the labeled peptides were detected by autoradiography. The arrows indicate the positions of the corresponding radiolabeled proteins. The numbers in parentheses indicate the lanes in which particular OT subunits are located. α, heptapeptide was used for photocatalysis. β, pentapeptide was used for photocatalysis. After elution of each sample from the immunoprecipitate, one half of the sample was directly applied to SDS-PAGE (lanes 1, 3, and 5), whereas the other half was subjected to Endo H digestion (lanes 2, 4, and 6).
autoradiography. This photoprobe labeled two proteins of the OT complex (Fig. 1a, lanes 2 and 3). Based on the molecular mass, the upper radiolabeled band could be either Ost1p or Stt3p, and the lower band could be Ost3p. Although the calculated molecular mass for Stt3p is 78 kDa, it was found that it migrates anomalously (runs between 60 and 70 kDa) and always as a diffuse band, and therefore it is hard to separate Ost1p from Stt3p (33, 35). To clarify these issues, we used microsomes prepared from strains that expressed either Ost1HAp or Stt3HAp for photolabeling experiments. The triple HA tag has a mass about 3 kDa, and we can detect the migration change by adding it to the C terminus of either Ost1p or Stt3p. When using microsomes prepared from the OST1HA strain, the upper radiolabeled band did not change its mobility, whereas the lower band increased its mobility. In comparison, when microsomes from an STT3HA containing strain were used, the upper radiolabeled band decreased its mobility, whereas the lower band migrated as it did in the OST1HA construct. These results clearly established that the upper radiolabeled band was Stt3p and that the lower radiolabeled band was Ost3p. It seems that when epitope-tagged Ost3p was replaced by the other epitope-tagged subunits for immunoprecipitation, less Ost3p was precipitated (lanes 1 and 4). Similar results were obtained by Karaoglu et al. (33).

This heptapeptide was designed to be a bifunctional photoprobe with the hope that it could cross-link two nearby OT subunits. Based on our results this obviously was not the case. If this peptide cross-linked two proteins together, we should detect a radiolabeled band that migrated at a position equal to the sum of the two polypeptides. We conclude that the photoprobe is acting as a monofunctional probe, although there are two photoreactive groups available. Because the excitation time of the photoprobe is short (0.8–1.2 μs in the presence of a suitably oriented C–H bond) (28), it is unlikely that two photoreactive groups would be excited at the same time. The excited state readily relaxes to the ground state if it does not encounter a hydrogen donor with the appropriate geometry. Once one Bpa is cross-linked to one protein, the other one might not be positioned optimally for photolyzing and linking to another protein.

We also prepared a pentapeptide bifunctional photoprobe in which one of the Bpa residues was placed between Asn and Thr, the same position as in the original tripeptide (Table I, peptide I), and another Bpa was located at the C terminus (Table I, peptide III). This peptide was found to be a good substrate for OT. Microsomes from an OST3HA strain were used for photoactivation followed by nondenaturing immunoprecipitation. Three radiolabeled bands were detected on the autoradiogram (Fig. 1b, lane 3). Based on the molecular mass and the characteristics of the bands, we assumed that they were Stt3p, Ost1p, and Ost3p. This was confirmed by using microsomes prepared from OST1HA and STT3HA strains for photoactivation. When using Ost1HAp microsomes for photoactivation, the upper band (Stt3p) did not change its mobility compared with using the Ost3HAp microsomes. But the middle band (Ost1HAp) decreased its mobility because of the addition of the triple HA tag, and the lower band increased its mobility because it no longer had a triple HA tag (Ost3p) (Fig. 1b, lane 1). Similarly, when using Stt3HAp microsomes for photoactivation, the upper band (Stt3HAp) migrated more slowly com-

![Sequence alignment of the C-terminal lumenal domain of Stt3p.](http://www.jbc.org/)

**FIG. 2.** Sequence alignment of the C-terminal lumenal domain of Stt3p. The identification of the human, Drosophila, and C. elegans Stt3p was obtained using BLAST version 2.0. Alignments were performed using the Macvector program. The identical amino acids are boxed and shaded, whereas the conservative replacements are only boxed. The identity was over 50%. Gaps are indicated by dashes.
pared with using the Ost3HAp microsomes, whereas the lower band (Ost3p) migrated more quickly, and the middle band (Ost1p) did not change its mobility (Fig. 1b, lanes 2, 4, and 6). By comparing lanes 2, 4, and 6, it is clear that these three radiolabeled bands are Stt3p, Ost1p, and Ost3p.

It is obvious that this pentapeptide also did not act as a bifunctional photoprobe, that is, it did not label any two polypeptides simultaneously. However, the fact that it became linked to Ost1p was consistent with the photolabeling of Ost1p when the tripeptide was used because one of the two Bpa residues in the pentapeptide is located in the position between Asn and Thr. It is of interest that the probe with Bpa at the C terminus of the pentapeptide labeled two different proteins. We speculate that this Bpa is probably located at the interface of Stt3p and Ost3p. These two subunits are likely to be near to each other because it is well established that they exist in one subcomplex (Ost3p-Ost4p-Stt3p) (33, 35, 36). It is not surprising that Bpa could label both Stt3p and Ost3p because the photoreactive group is free to rotate.

Block Mutations of Stt3p—From the previous results it is clear that both Stt3p and Ost3p are in the vicinity of Ost1p in the OT complex. This conclusion is supported by the observation that upon depletion of Stt3p, both Ost1p and Ost3p disappear from the purified OT complex, whereas Wbp1p and Swp1p are still present (17). Because Stt3p is the most conserved subunit of OT complex, it is reasonable to speculate that it contains the recognition element and/or catalytic domain for glycosylation. Ost3p is not an essential subunit and thus is not likely to carry out a function absolutely required for glycosylation.

The subunit that contains the recognition element and/or catalytic domain for glycosylation must be essential, and mutations in the recognition element and/or catalytic domain would be expected to cause lethality or a severe growth defect. However, if the mutant protein does not get incorporated into the OT complex, we cannot determine whether it is a functional mutation. If it does get incorporated and if the mutation is not lethal, the OT activity would be expected to be dramatically decreased in the mutant. Most importantly, the mutant would be expected to cause a marked decrease in the labeling of Ost1p by the tripeptide Asn-Bpa-Thr. We asked whether Stt3p meets all of these criteria. A search for homologous proteins in the data bases showed that yeast Stt3p shares high homology with proteins in human, Drosophila, and C. elegans (greater than 50% identity over a region of 700 amino acid residues). It even shares homology with a protein in archaeabacteria (35). It is remarkable that the identity shared among these proteins is not restricted to one region but rather is spread out over the entire polypeptide. Sequence analysis also demonstrated that the secondary structure predicted for these homologous proteins is quite similar (17).

Fig. 2 illustrates the very high conservation of the luminal domains of Stt3p among different species. It is well established that the active site of OT is located in the ER lumen, and for this reason we focused our attention on the luminal domain of Stt3p in the ER (37). First, on the basis of high conservation, we prepared 21 block mutants consisting of 2–5 residues replaced by Ala residues in an epitope-tagged Stt3p construct (Table II). These mutants were transformed into an stt3 null strain containing wild type STT3 on a URA3 plasmid. First, we used the plasmid shuffling procedure to ask whether these Ala mutations caused loss of function in these mutant stt3p. Out of the 21 mutants, six of them properly functioned and were not further studied. Of the remainder, 14 mutants were lethal, and one mutant was temperature-sensitive (Table II). Next, we determined which of these block mutant proteins were incorporated into the OT complex in cells because, as noted above, mutants that do not make stable proteins or proteins that cannot be incorporated into the OT complex are of very limited value. It is important to note that the 14 lethal mutants also contain the wild type STT3 gene to support the growth, but the temperature-sensitive mutant contained only the mutant.

### Table II

| Mutants       | Growth phenotype |
|---------------|------------------|
| 487–488AA     | normal           |
| 494–498AAAAAA | lethal           |
| 500–504AAAAAA | lethal           |
| 506–507AA     | normal           |
| 510–512AAAAA  | lethal           |
| 516–519AAAAA  | lethal           |
| 529–532AAAAA  | lethal           |
| 533–536AAAAAA | lethal           |
| 537–540AAAAAA | lethal           |
| 541–543AAAAA  | lethal           |
| 547–548AAAA   | normal           |
| 554–556AAAA   | t.s.             |
| 557–599AAAA   | normal           |
| 565–568AAAAAA | lethal           |
| 573–575AAAAA  | lethal           |
| 582–584AAAAAA | lethal           |
| 585–587AAAAA  | lethal           |
| 592–594AAAAA  | normal           |
| 612–613AAAAA  | normal           |
| 667–668AAAA   | normal           |
| 678–680AAAA   | lethal           |
| W516Y         | t.s.             |
| W517Y         | lethal           |
| D518E         | lethal           |
| G520A         | t.s.             |
| I593A         | t.s.             |

a The position of amino acid residues replaced by Ala in Stt3p.
b Growth phenotype was determined by 5-fluro-orotic acid selection. t.s., temperature-sensitive.
stt3p. All of the mutant constructs and the wild type control construct of Stt3p contained the HA epitope-tag at their C terminus. Detergent extracts were used for immunoprecipitation with anti-HA antibodies, and the precipitated proteins were fractionated on SDS-PAGE and then followed by Western blot analysis. Using the antibodies available to us, we found that mutants 516–519AAAA, 592–594AAAA, and 554–556AAAA were assembled into the OT complex and co-immunoprecipitated other OT subunits, including Ost1p, Wbp1p, and Swp1p (Fig. 3). For the temperature-sensitive mutant 554–556AAAA, the mutant protein was incorporated into the OT complex at both 25 and 37 °C. Of the remainder, some stt3p proteins were not immunoprecipitated well themselves, and others did not co-immunoprecipitate other OT subunits; these mutants were not further studied.

These three block mutants (516–519AAAA, 592–594AAAA, and 554–556AAAA) cannot function in the cells even though they are incorporated in the OT complex. It is very interesting that one of the three block mutants, 516–519WWDYG, is in a region that shares the highest sequence similarity among eukaryotic Stt3p and the archaeal proteins (35) (Fig. 2). Actually, almost all residues of 516–520WWDYG are conserved in all the archaeabacteria proteins (Initially, Gly520 was not included in this block mutation because it was known that G520D and G520S were temperature-sensitive mutants, but later the G520D mutant was included when preparing other point mutants based on the block mutations). It seems reasonable to presume that this level of identity reflects functional importance.

In our earlier study, it was shown that although when using the Asn-Bpa-Thr tripeptide for photolabeling Ost1p was spe-
specifically labeled, this subunit did not contain the peptide glycosylation recognition and/or catalytic domain. Stt3p is in the proximity to Ost1p and is the most conserved subunit of OT. Therefore, if particular residues in Stt3p are involved in glycosylation site recognition and/or the catalytic site, mutation of these residues should abolish labeling of Ost1p by Asn-Bpa-Thr. Microsomes were prepared from these three stt3 block mutants, and photolabeling was carried out using radiolabeled Asn-Bpa-Thr. The results clearly demonstrated that mutant 516–519AAAA and mutant 592–594AAAA had drastically reduced labeling of Ost1p (0.115 and 0.200 compared with the wild type control), whereas mutant 554–556AAAA had a level of labeling one half of the wild type (Fig. 4, a and b, and Table III). We speculate that 516–520WWDYG forms the binding region for the glycosylation recognition site or serves as the active site of the enzyme. Clearly, when these residues are mutated, Asn-Bpa-Thr cannot be recognized or cannot be positioned at the correct site for glycosylation, and therefore the photolabeling of Ost1p is greatly reduced. The residues 554–556EEK and 592–594RIS are probably not within the active site but instead may be involved in positioning the active site of the enzyme. When they are mutated, a modified enzyme conformation might reduce the binding of the substrate, and therefore the photolabeling of Ost1p would be greatly reduced.

Single Amino Acid Mutations of Stt3p—Because these three block mutants had important characteristics, we undertook to prepare 10 single amino acid mutants in which each residue was replaced either by Ala or by Asp in an epitope-tagged STT3 construct (Table IV). Five mutants derived from changes of the most conserved region, 516–520WWDYG, displayed the most severe growth defects. Two mutations caused lethality, and the other three were temperature-sensitive. Three point mutations derived from the temperature-sensitive block mutant 554–556EEK caused no growth phenotype, and we did not pursue additional studies with them. As to the block mutant 592–594RIS, we prepared only two single mutations; Ser594 was not included because this residue is not highly conserved. Although I593D was a temperature-sensitive mutant, R592A had no growth phenotype, and it was not studied further.

We performed a spotting assay to analyze the growth phenotype of the four temperature-sensitive mutants. As shown in Fig. 5, the growth rate for these cells was wild type > Y519A > G520D > I593D > W516A. Next, we examined the incorporation of the six single-residue mutant proteins into the OT complex. The two lethal mutants also contained the wild type STT3 gene to support the growth, but the temperature-sensitive mutants had only their own copy of stt3. All of the mutant constructs and the wild type control contained the HA tag at their C terminus for immunoprecipitation and could co-immunoprecipitate other OT subunits except G520D (part of the data are shown in Fig. 6 below, also see Table IV). Protein made from the G520D construct was comparable in amount with the wild type, but it could not co-immunoprecipitate other OT subunits well. Interestingly, in genetic screens aimed at the identification of components required for the process of N-linked glycosylation in the ER, Markus Aebi's group identified more than 50 stt3 alleles, and four of those had a temperature-sensitive phenotype (35). Surprisingly, three of the four mutations affected the same amino acid residue, which was Gly520; two of these were G520D, and one was G520S.

Although these single-residue mutants are incorporated into the OT complex, we wanted to determine whether they could support the photolabeling of Ost1p when using the Asn-Bpa-Thr photoprobe for photoactivation. All of the temperature-sensitive mutants had lower Ost1p photolabeling. Of the two lethal mutants, W517A and D518A exhibited residual labeling when Trp 517 and Asp 518 were mutated, the cells could not survive without a copy of the wild type gene, and there was only a low level of Ost1p labeling. Residues 516–520WWDYG may well function as the catalytic site for glycosylation. Among these five residues, Trp517 and Asp518 are most important because mutation of these two caused lethality. When Trp517 and Asp518 were mutated, the cells could not survive without a copy of the wild type gene, and there was only a low level of Ost1p labeling.

Residues 516–520WWDYG may well function as the catalytic site rather than merely as the recognition site to which Asn-Xaa-Thr/Ser binds. In the two mechanisms proposed earlier for the OT enzymatic reaction (see below), there is a base in the enzyme active site that extracts a proton from either Asn or Thr, thus increasing the nucleophilicity of the carboxamide nitrogen so it can react with the electrophilic lipid-linked oligosaccharide. In this case, Asp518, with the Asp fully ionized, is a perfect candidate for such a base in the enzyme active site.

In addition to changing these residues to Ala or Asp, we also changed these residues to somewhat similar residues (Table II). W517Y and D518E were still lethal mutants, and W516Y was temperature-sensitive as well. Therefore, an aromatic ring is not sufficient at position 516 or 517, and the enzyme probably requires the hydrophobicity of the Trp. Trp often serves as a hydrophobic cluster in the conformational properties of proteins, and mutation of this residue could perturb the original conformation (38–40). Surprisingly, Glu518 could not replace...
the Asp at all; the extra CH₂ renders it nonfunctional. This further demonstrates the importance of this Asp, because it is not only the negative charge but also the chain length that is important. These findings suggest that Asp⁵¹⁸ could be in the catalytic site.

We also measured the OT activity of these single-residue mutants. We could not directly assay the lethal mutants because they have a wild type copy of STT3. First, the expression of Ost1p, Wbp1p, and CPY was examined in the temperature-sensitive mutants because it has been shown that these proteins are glycosylated (Fig. 6). Ost1p contains four potential glycosylation sites, and usually three or four sites are utilized (11, 26). Wbp1p contains two potential glycosylation sites, and one or two are glycosylated (7, 26). In mature CPY all four glycosylation sites should be occupied (41). It is clear that the proteins detected by their corresponding antibodies migrated as ladders. These ladders are the different glycoforms of the proteins because they all shifted to one band after deglycosylation with protein glycanase F or Endo H (data not shown for Ost1p and Wbp1p). It is obvious that Ost1p, Wbp1p, and CPY were underglycosylated in the mutant cells.

In addition, direct in vitro OT activity assays were performed on the single-residue temperature-sensitive mutants. Compared with the wild type control, extracts prepared from the stt3 mutants displayed much lower OT activity (Table IV). This result correlates well with the in vivo underglycosylation of the glycoproteins in these mutants. Clearly, these residues are important in the function of OT, and therefore we investigated their apparent affinity for the substrates. As shown in Fig. 7, the level of glycosylated peptide formation was dependent on the substrate concentration. However, at low substrate concentrations, the extracts from the mutant strains exhibited much less product formation when compared with the extract from the wild type strain. Only at high substrate concentrations were the glycosylated products formed in the mutants comparable with the amount formed in the control. Because we had a limited quantity of the radiolabeled substrates, we could not perform OT assays using even higher substrate concentrations.

**Fig. 7.** Assay of the temperature-sensitive mutants of Stt3p for OT activity. Yeast spheroplasts were prepared from each mutant, and [³H]Ac-Asn-Bpa-Thr-Am was used as the substrate. The reaction was carried out as described under “Experimental Procedures.” In each reaction, the amount of spheroplasts was the same, and the concentration of the radiolabeled peptide varied. Each point on the plots is the average of duplicates. This assay was performed three separate times, and all assays yielded similar plots.

**Fig. 8.** Highly diagrammatic representation of the labeling observed with the three photoprobes. The proteins that became labeled are shown in the boxes. Except in the case of Ost1p, we do not know which Bpa residue at the distal positions labeled Stt3p or Ost3p in panel B. A, ¹²⁵I-bh-Asn-Bpa-Thr-Am. B, ¹²⁵I-bh-Bpa-Ala-Asn-Ala-Thr-Ala-Bpa-Am. C, ¹²⁵I-bh-Asn-Bpa-Thr-Ala-Bpa-Am.
But based on the data obtained these mutant proteins may have a higher $K_m$ for the peptide substrates.

Conclusions—OT catalyzes the formation of an N–C bond between the amide nitrogen of the asparagine side chain and the C1 position of the N-acetylglucosamine residue of the Dol-PP-oligosaccharide. Although Dol-PP is a reasonable leaving group, it is not clear how the nucleophilicity of the asparagine side chain is enhanced so that it can displace Dol-PP and form the $N$-glycosidic bond. Besides the asparagine residue, the requirement for a hydroxyl amino acid implies a direct role for the hydroxyl group in catalysis. Buse and Legler (42) proposed a mechanism in which the hydroxyl group of the serine or threonine in the peptide acts as a hydrogen acceptor, with one of the carbamoyl hydrogen atoms of the asparagine side chain as the donor. This is proposed to occur because a basic residue at the enzyme active site abstracts a proton from the hydroxyl group of serine or threonine, and this, in turn removes a proton from the amide. The resulting strong nucleophile would then displace the dolichol pyrophosphate from the C1 position of the N-acetylglucosamine containing oligosaccharide.

Besides its consensus sequence requirements, it seems that conformation of the peptides is important because many Asn-Xaa-Thr/Ser sequences are not glycosylated following translocation and translocation into the ER lumen (2, 43). The conformation that the Asn-Xaa-Thr sequence can adopt can be either the $\beta$-turn or the Axx turn. The $\beta$-turn is characterized by a hydrogen bond between the threonine amide and the carbonyl immediately preceding the asparagine. As pointed out by Imperiali and Shannon (44), the Axx turn involves a hydrogen bond between the carbamoyl oxygen of asparagine and the backbone amide and the side chain hydroxyl of threonine or serine, which both act as hydrogen donors. When synthetic cyclic peptides were tested it was found that peptides constrained into $\beta$-turns are not substrates for OT, whereas peptides with Axx turn conformations have enhanced affinity for the enzyme (45). Accordingly, Imperiali et al. (46) proposed a model in which the basic residue at the active site of the enzyme abstracts a proton from the $\beta$-turn and subsequently induces tautomerization of the carbamoyl to an imidol. This imidol can act as a reactive nucleophile for displacement of the dolichol pyrophosphate from the electrophilic dolichol-linked oligosaccharide donor to yield the glycosylated product and dolichol pyrophosphate. This model provides a structural explanation for the sequence specificity of the acceptor substrates, because sequences such as Gln-Xaa-Thr(Ser) cannot serve as oligosaccharide acceptors because they cannot adopt an analogous Gln turn conformation. In both models, the presence of an active site base is a central element because depletion is essential for the formation of a competent nucleophile species.

The involvement of the Axx turn in the glycosylation reaction proposed by Imperiali is supported by the observation that cyclized peptides that can adopt the Axx turn are good substrates of OT (45, 46). Given these findings and the results on Stt3p presented in this paper, and on Ost1p,3 we propose that the nascent polypeptides that are being elongated pass by Stt3p, and if they contain an Asn-Xaa-Thr(Ser) (with an Axx turn), they fit into a cleft in Stt3p. This is shown in the diagram in Fig. 8, which summarizes the labeling observed with the three different photoprobe. This cleft is proposed to represent the recognition domain that would contact the backbone of the substrate only when it is in the Axx turn conformation. The sequence WWDDYG in Stt3p is either part of the recognition domain to contact the Axx turn or it could be part of the active site. If it is the latter case, it must be in close physical proximity to the glycosylation site recognition domain of Stt3p, thereby allowing the ionized form of D (we found that the longer side chain E is not functional), in conjunction with a base, to extract a proton from the amide side chain. In this way the amide could function as a nucleophile in the attack on the Dol-PP-oligosaccharide. In the Axx turn, the photoreactive side chain of Bpa in the Xaa position would be oriented outward, and it therefore would label the nearby protein Ost1p rather than Stt3p.

In contrast, when a heptapeptide with Bpa at two positions distal to Asn-Xaa-Thr/Ser is used, both Stt3p and Ost3p are labeled (Table I and Fig. 8). Finally, when a pentapeptide with Bpa at one of these distal locations and one located at the Xaa position are used, Ost1p, Ost3p, and Stt3p are labeled. These observations are internally consistent with an Axx turn extending into a cleft where the recognition and/or active sites of Stt3p are located, and the more distal portions of the substrate are in close opposition to Ost1p and Ost3p. This model is also consistent with the observation that down-regulation of Stt3p leads to a loss of Ost1p and Ost3p in the purified OT complex (17).

Finally, this model is supported by the recent unpublished observation3 that Ost1p and Stt3p can be chemically cross-linked to each other. Clearly, these models for the action of Stt3p in the glycosylation process are based on a variety of observations from various experiments in different laboratories. Only when it is possible to obtain the three-dimensional structures of these proteins of the OT complex will it be possible to test the validity of them.

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