Selective Control of Oligosaccharide Transfer Efficiency for the N-Glycosylation Sequon by a Point Mutation in Oligosaccharyltransferase*\(^1\)**

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Asn-linked glycosylation is the most ubiquitous posttranslational protein modification in eukaryotes and archaea, and in some eubacteria. Oligosaccharyltransferase (OST) catalyzes the transfer of preassembled oligosaccharides on lipid carriers onto asparagine residues in polypeptide chains. Inefficient oligosaccharide transfer results in glycoprotein heterogeneity, which is particularly bothersome in pharmaceutical glycoprotein production. Amino acid variation at the X position of the Asn-X-Ser/Thr sequon is known to modulate the glycosylation efficiency. The best amino acid at X is valine, for an archaeal *Pyrococcus furiosus* OST. We performed a systematic alanine mutagenesis study of the archaeal OST to identify the essential and dispensable amino acid residues in the three catalytic motifs. We then investigated the effects of the dispensable mutations on the amino acid preference in the N-glycosylation sequon. One residue position was found to selectively affect the amino acid preference at the X position. This residue is located within the recently identified DXKXXX(M/I) motif, suggesting the involvement of this motif in N-glycosylation sequon recognition. In applications, mutations at this position may facilitate the design of OST variants adapted to particular N-glycosylation sites to reduce the heterogeneity of glycan occupancy. Thus, a mutation at this position led to 9-fold higher activity relative to the wild-type enzyme, toward a peptide containing arginine at X in place of valine. This mutational approach is potentially applicable to eukaryotic and eubacterial OSTs for the production of homogenous glycoproteins in engineered mammalian and *Escherichia coli* cells.

N-linked glycosylation is the most ubiquitous posttranslational protein modification in eukaryotes and archaea, and in some eubacteria (1–4). It plays an important role in determining the biological activities of glycoproteins as well as the medicinal efficacy of many pharmaceutical recombinant glycoproteins. The covalent attachment of an oligosaccharide occurs on the side chain of an asparagine residue in a polypeptide chain, within the Asn-X-Ser and Asn-X-Thr sequences, where X is any amino acid except for proline (5, 6). About one-third of the potential N-glycosylation sequons are not glycosylated in extracellular proteins (7–9). This fact indicates that the presence of a consensus sequence is essential but not sufficient for N-glycosylation. The percentage of proteins modified at a particular sequon is referred to as “site occupancy.” A recent comprehensive proteome analysis of mouse tissues and blood plasma revealed that more than 98% of N-linked glycosylated peptides derived from glycoproteins were not found in their unmodified form, indicating the high occupancy of N-glycosylation sites (10). Thus, for most N-glycosylation sequences *in vivo*, the N-glycan attachment is robust, resulting in a homogeneously glycosylated (100% occupancy) or unglycosylated (0% occupancy) site.

In contrast, for some recombinant glycoproteins produced in cultured cells, the N-glycan attachment is variable, resulting in a mixture of various glycoforms, including fully, partially, and unglycosylated protein species (11). This variation in glycan occupancy is referred to as “macroheterogeneity.” Because the glycan attachment enhances solubility, improves folding, facilitates secretion, and increases the *in vivo* half-life of glycoproteins, glycosylation isoforms of pharmaceutical glycoproteins, such as Antithrombin III and tissue-type plasminogen activator, have different medicinal efficacies within humans (12, 13). Therefore, controlling the macroheterogeneity of recombinant glycoproteins is vital in therapeutic glycoprotein production.

Oligosaccharyltransferase (OST)\(^2\) catalyzes the transfer of preassembled oligosaccharides on lipid carriers (lipid-linked oligosaccharides (LLO)) onto asparagine residues in polypeptide chains. The site occupancy is determined by the action of OST in eukaryotic cells. Although little information is available, the situation is probably similar in prokaryotic cells. Thus, the critical factors that affect the site occupancy are the supply of the oligosaccharide donors (LLO), the properties of the polypeptide acceptors, and the activity of the OST enzyme. First, in cultured cells overexpressing a recombinant glycoprotein, the limited supply of LLO induces variable site occupancy. The addition of exogenous intermediate substances is expected to

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increase the level of N-glycosylation, but it is not always effective because of the complexity of the biosynthetic pathway of LLO (11). Second, the manipulation of the amino acid sequence within a sequon and its flanking residues could improve the efficiency of N-glycosylation. The Asn-X-Thr sequon is always a better acceptor than the Asn-X-Ser sequon (13–15). The amino acid at the X position and those at the other positions adjacent to the sequon also influence the glycosylation efficiency (14–19). Thus, changing the amino acid sequence within and near the N-glycosylation site generating macromethod involves the custom-tailoring of an OST enzyme by mutagenesis. This provides an ideal solution for a specified target protein without manipulation of its amino acid sequence.

Oligosaccharyltransferase is a multisubunit membrane protein complex in higher eukaryotes (2) but a single-subunit membrane protein in lower eukaryotes, archaea, and eubacteria (20–22). The catalytic subunit of the OST enzymes has a common ancestor but is referred to differently among the three domains of life, as STT3 in eukaryotes, AglB in archaea, and PglB in eubacteria. The primary sequences (600 to 1000 residues) of the STT3/AglB/PglB proteins share a common architecture (23, 24). There is a multispan transmembrane region in the N-terminal half of the primary sequence, and the C-terminal half forms a globular domain (Fig. 1).

The sequence alignment of the STT3/AglB/PglB protein family members revealed two conserved motifs. A five-residue motif, WWDYG, resides in the C-terminal globular domain, whereas a diacidic motif, DXD or EXD (X denotes any amino acid residue), is found in the first luminal/extracellular loop of the N-terminal transmembrane region. Mutagenesis studies demonstrated the essential roles of these two motifs in the OST activity of the STT3s from yeast and Leishmania major and PglB from Campylobacter jejuni (21, 25–29). In addition to the two motifs, the structure-based sequence alignment revealed a third conserved motif (21). The third motif was classified into three groups, according to the different consensus patterns, DXXXXXX(M/I), DXXMXXX(K/I), and MXLXXX(1/V/W), which were named DK, DM, and MI, respectively (27). Eukaryotic STT3 proteins exclusively contain the DK motif, whereas eubacterial PglB proteins only contain the MI motif. In contrast, archaeal AglB proteins contain either the DK, DM, or MI motif. In vivo mutagenesis studies of the STT3 proteins from yeast and L. major revealed the requirement of the DK motif for survival (21, 28). An in vitro mutagenesis study of the C. jejuni PglB protein also confirmed the involvement of the MI motif in the activity (27).

We developed an in vitro OST assay using recombinant AglB expressed in Escherichia coli membrane fractions. The AglB protein from a hyperthermophilic archaeon, Pyrococcus furiosus, was used to perform a systematic mutagenesis study. We identified a residue position where amino acid replacement induced specific changes in the amino acid preference at the X position of the Asn-X-Thr sequon in peptide substrates. This study highlights the feasibility of creating a single point mutation in OST to optimize its activity toward a given N-glycosylation sequon, which would eventually lead to reduced macromethod involves the custom-tailoring of an OST enzyme by mutagenesis. This provides an ideal solution for a specified target protein without manipulation of its amino acid sequence.
transferred to a plastic tube and dried in a SpeedVac concentrator. Eight microliters of buffer (50 mM Tris–HCl (pH 7.5) containing 1 mM DTT, 10 mM MnCl₂, 0.02% Tween 20) were added, and the assay tube was sonicated in a bath-type sonicator (120 W) for 5 min. One microliter of 30 μM TAMRA-peptide solution and 3 μl of the E. coli membrane fraction containing the full-length AglB or its mutants was added. The reaction mixture (total 12 μl) was incubated at 65 °C for 1 h. The reaction was stopped by adding 2.4 μl of 5× SDS sample buffer and heating at 95 °C for 1 min. The fluorescence images of the SDS-PAGE gel were recorded with an LAS-3000 multicolor image analyzer (Fuji Photo Film) and were quantified using the Image-Gauge software (Fuji Photo Film). The relative acceptor efficiency of an individual sequon in the peptide library experiments is defined as the ratio of the amount of each glycopeptide to the total amount of the 19 glycopeptides. The changes in the amino acid preferences of the OST mutants were evaluated by a correlation analysis between the relative acceptor efficiencies of the wild-type AglB and its mutants.

RESULTS

Overexpression and Purification of Recombinant AglB—The DNA encoding the full-length AglB gene was amplified from P. furiosus genomic DNA and cloned into the vector pET15b with a C-terminal His tag. The protein was expressed in the membrane fraction of E. coli cells, solubilized with 1% n-dodecyl-β-D-maltoside, and purified by nickel affinity chromatography. To measure the amount of the AglB protein, we performed Western blotting and infrared imaging with fluorescently labeled secondary antibodies (supplemental Fig. S1). The details of the experimental results are provided under “Supplemental Information.”

In Vitro Mutagenesis Study of the Three Catalytic Motifs—To address the role of the three conserved motifs, WWDYG, DXD, and DK, alanine-scanning mutagenesis was performed. The positions of the mutations in the tertiary structure are shown in Fig. 1. All of the mutant proteins were expressed at levels similar to that of the wild-type protein (supplemental Fig. S1D). The oligosaccharyltransferase activity was measured in the presence of the LLO prepared from P. furiosus cells as the oligosaccharyl donor and a synthetic eight-residue peptide containing the N-glycosylation consensus as the acceptor (Fig. 2A). The peptide consists of the optimized amino acid sequence (14) and contains a fluorescent dye at the N terminus. The generated glycopeptides were separated from the unmodified peptides by SDS-PAGE and quantified by fluorescence imaging (30). The specific activity was defined as the amount of glycopeptide formed (fmol) per hour per unit amount of protein defined by infrared imaging (see details in supplemental Fig. S2).

The substitution of each aspartate residue in the DXD and WWDYG motifs led to a nearly complete loss of the activity (Fig. 2B). The substitution of each of the tryptophan residues in the WWDYG motif also impaired the enzymatic activity. The mutations of the tyrosine and glycine in the WWDYG motif had an inhibitory effect. The mutations of the three signature residues in the DK motif, Asp-571, Lys-574, and Ile-578, also had an inhibitory effect on the OST activity. These results confirmed the vital contribution of the three conserved motifs to the catalytic function of the archaeal OST.

![FIGURE 1. Structure of the P. furiosus OST/AglB protein and positions of the mutated residues. The predicted membrane topology of the N-terminal transmembrane region and the crystal structure (2ZAG and 2ZAI) of the C-terminal globular domain of the P. furiosus AglB are shown. The predicted positions of the DXD motif and Asp-167 are indicated by yellow stars. The Asp-167 residue belongs to another previously unrecognized diacidic motif, GXXD/E (supplemental Fig. S4). The mutated amino acid residues are shown with the side chains in the close-up view of the active site of the OST/AglB protein. WWDYG motif, yellow; DK motif, green; other residues on the long, kinked helix, orange.](Image 60x612 to 288x733)

![FIGURE 2. Effects of the alanine mutations on the oligosaccharyltransferase activity. A, schematic representation of the oligosaccharyltransferase activity assay. The OST enzyme requires an LLO as the donor and an oligopeptide containing the N-glycosylation sequon as the acceptor. The P. furiosus LLO is predicted to be a dolichol-pyrophosphate-linked glycan. Hexagons, P. furiosus N-glycan consisting of seven monosaccharide residues (21); circles, pyrophosphate group; rectangles, dolichol; φ, TAMRA dye. The glycopeptide product was separated from the unmodified peptide by SDS-PAGE and quantified by fluorescence imaging. B, alanine mutants in the DXD motif, at Asp-167, in the WWDYG motif, and within and near the DK motif. The alanine residue in the wild-type sequence was replaced by a glycine residue. The peptide substrate TAMRA-Ala-Pro-Tyr-Asn-Val-Thr-Lys-Arg contained the optimized sequence for P. furiosus AglB (14). The specific OST activity of the wild-type AglB was defined as 1. Data were compared with the wild type by an unpaired two-tailed t test, assuming unequal variance; *, p < 0.01; §, p < 0.05. The error bars represent the mean ± S.D. of three independent experiments.](Image 464x650 to 493x722)
Point Mutation at Position 574 Affects the Amino Acid Preference for the X Position of the Glycosylation Sequon—We compared the substrate specificity of the mutant AglB with that of the wild-type AglB by examining the amino acid preference at the X position of the Asn-X-Thr sequon in the peptide substrates. We prepared a peptide library, sharing a common sequence, Cys-Arg-Gly-Asn-X-Thr-Ala-Arg (14). A fluorescent dye was appended to the side chain of the N-terminal cysteine for detection and quantification. The relative acceptor efficiency of different N-glycosylation sequences was determined for the wild-type enzyme (Fig. 3A). There were wide variations in the preference among the amino acids at X. A sequon containing proline was inactive, whereas a sequon with valine served as the most favored substrate. The same peptide library experiment was performed using the G515A mutant for comparison, and we found a similar tendency in the relative amino acid preference. In fact, the correlation plot between the wild-type enzyme and the G515A mutant shows a good linear relationship (Fig. 3A), although the G515A mutation caused a 40% reduction in the specific activity (Fig. 2B). We then tested nine other mutations that were dispensable for the OST activity. Among them, only the K574A mutant changed the amino acid preference significantly (Fig. 3B). A closer look at the correlation plot revealed that the mutation-induced changes in the preference were selective to arginine, lysine, and glutamate residues at the X position (Fig. 3C). The correlation plots for the other mutants are provided in supplemental Fig. S3.

We then substituted Lys-574 with six different amino acids other than alanine. The effects on the amino acid preference at the X position were similar to the K574A mutants (Fig. 4): increased efficiency for arginine and lysine and decreased efficiency for glutamate. The enhanced efficiency for arginine at the X position suggests that the K574 mutants may have a higher activity than the wild-type enzyme toward peptides containing the Asn-Arg-Thr sequon. As expected, four mutants had a higher activity on the Asn-Arg-Thr sequon than the wild-type enzyme (Fig. 5B). In contrast, all mutants had a lower specific activity than the wild type on the original Asn-Val-Thr sequon (Fig. 5A).

DISCUSSION

Mutagenesis Study of P. furiosus AglB—The sequence homology suggests that three conserved motifs are required for the OST activity. This expectation has been proven right for the eukaryotic STT3s and the eubacterial PglB by mutagenesis studies (21, 25–29). Here, we verified the contribution of these motifs to the OST activity of an archaeal AglB protein. We set up an in vitro OST assay using P. furiosus AglB expressed in the membrane fractions of E. coli cells (supplemental Figs. S1 and S2). The detection of the OST activity using the heterologously expressed AglB confirmed that the AglB protein is solely responsible for the OST activity and that neither posttranslational modifications nor other additional subunits are necessary for the OST activity. This finding guarantees the future
installation of the \textit{P. furiosus} AglB into artificial N-glycosylation machinery engineered in \textit{E. coli}.

The almost complete loss of the OST activity by the mutations in the DAD and WWDYG motifs confirmed the essential roles in the archaeal OST activity (Fig. 2B). The alanine mutation of Asp-167 also impaired the OST activity (Fig. 2B). The Asp-167 residue belongs to another previously unrecognized diacidic motif, GXXDX(D/E) (supplemental Fig. S4). In good agreement, the alanine mutations in the corresponding region (G\textsubscript{163}SYD\textsubscript{168}) in yeast STT3 generated a lethal phenotype (31). Each alanine mutation of the three residues that define the DK motif reduced the OST activity, whereas the other mutations, except for Tyr-568 and Phe-575, within and flanking the DK motif retained nearly full activity (Fig. 2B). The results are in good accordance with the three-dimensional structure of the AglB protein. The three signature residues face the same side of the kinked helix, and the side chains are in close proximity to the essential WWDYG motif (Fig. 1).

FIGURE 5. \textit{P. furiosus} OST was adapted for a non-optimal N-glycosylation sequon by the point mutations at position 574. A, the specific OST activities of the point mutants at position 574 were compared. The peptide sequence was the same as that used in Fig. 2. B, the same assay was performed using a different peptide with arginine at the X position in place of valine. The activity of the wild-type AglB was defined as 1. Data were compared with the wild type by an unpaired two-tailed \( t \) test, assuming unequal variance. *, \( p < 0.01 \); §, \( p < 0.05 \). The error bars represent the mean \( \pm \) S.D. of three independent experiments.

Tuning of Oligosaccharyltransferase Specificity

FIGURE 4. Comparison of the alanine amino acid preference at the X position of the N-glycosylation sequon between the wild-type AglB and the point mutations at position 574 to various amino acids. A, the plots show that all of the mutations had similar impacts on the amino acid preferences at the X position, i.e. improved efficiency for arginine and lysine and decreased efficiency for glutamate. B, the \( R^2 \) (squared correlation coefficient) values of the correlations were calculated before and after the omission of the marked amino acids. For example, \( R^2 \) is 0.504 for the K574A mutant, and it increased to 0.935 when the arginine, lysine, and glutamate were excluded from the calculation (as indicated by \(-\text{RKE}\)).
The present study suggests that the amino acid residue at the X position of the Asn-Val-Thr sequon is located in close proximity to the lysine residue in the DK motif (ii), that the highly conserved WWDYG is considered to be a nucleophilic region and would interact with the asparagine and threonine residues of the acceptor peptides (ii), and that the acidic residues in the DXD motif and Asp-167 are predicted to interact with the pyrophosphate group of LLO by binding to a divalent metal ion ($M^{2+}$) (ii).

![Figure 6. Schematic model of the catalytic site of the OST enzyme and the presumed functions of the catalytic motifs.](image)

Possibility of Customizing OST—The mutations at residue 574 substantially reduced the OST activity when the optimized peptide sequence containing the Asn-Val-Thr sequon was used as a peptide substrate (Fig. 5A). We measured the OST activity using a new peptide substrate with the Asn-Arg-Thr sequon (Fig. 5B). Four mutants displayed higher activity than the wild type. This exemplifies the potential of a single mutation in the AglB protein to optimize its activity toward a particular target N-glycosylation sequon. For practical applications, the present study provides a novel strategy to overcome the macroheterogeneity problem of recombinant glycoproteins.

The eukaryotic SFT3 proteins contain the DK motif, and thus the lysine residue in the motif is an obvious candidate for mutation. Recently, an innovative glycoprotein production system using the Campylobacter N-glycosylation machinery was reported (32). The key component of the machinery is Campylobacter Pg1B. The Pg1B protein possesses the MI motif in place of the DK motif (27). Mutations in the MI motif would affect the amino acid preference at the X position.

The common tendency of the changes in amino acid preference among the seven Lys-574 mutants of the P. furiosus AglB (Figs. 3C and 4) may preclude the flexible design of mutants targeted to a particular sequon with a variety of amino acid residues at the X position. We suggest that combinatorial mutation screening, based on the three signature sites in the DK/DM/MI motifs, may generate an optimized OST mutant for a particular substrate sequence. In fact, the alanine mutation of the third signature position of the DK motif, I578A, had a small effect on the amino acid preference at the X position (supplemental Fig. S3).