The properties of recombinant staphylokinase (Sak-STAR) expressed in Pichia pastoris cells have been determined. The single consensus N-linked oligosaccharide linkage site in SakSTAR (at Asn 28) of the mature protein) was occupied in approximately 50% of the expressed protein with high-mannose-type oligosaccharides. The majority of these glycans ranged in polymerization state from Man$_9$GlcNAc$_2$ to Man$_14$GlcNAc$_2$, with the predominant species being Man$_{10}$GlcNAc$_3$ and Man$_{11}$GlcNAc$_2$. Glycosylated SakSTAR (SakSTAR$_g$) did not differ from its aglycosyl form in its aggregation state in solution, its thermal denaturation properties, its ability to form a complex with human plasmin (hPm), the amidolytic properties of the respective SakSTAR-hPm complexes, or its ability to liberate the amino-terminal decapeptide required for formation of a functional SakSTAR-hPm plasminogen activator complex. However, this latter complex with SakSTAR$_g$ showed a greatly reduced ability to activate human plasminogen (hPg) as compared with the same complex with the aglycosyl form of SakSTAR. We conclude that glycosylation at Asn 28 does not affect the structural properties of SakSTAR or its ability to participate in the formation of an active enzymatic complex with hPm, but it is detrimental to the ability of the SakSTAR-hPm complex to serve as a hPg activator. This is likely due to restricted access of hPg to the active site of the SakSTAR$_g$-hPm complex.

Staphylokinase (Sak), a protein secreted by certain strains of Staphylococcus aureus, functions as a profibrinolytic agent by virtue of its ability to convert the zymogen human plasminogen (hPg) to its active serine protease form, plasmin (hPm). The Sak gene has been cloned and sequenced from serotype B and F bacteriophages sak42D and sak42D, respectively, and from the genome of a lysogenic strain of S. aureus (SakSTAR). The protein was initially expressed in relatively low yield and with variable amino-terminal processing in Escherichia coli (1, 4) and Bacillus subtilis (2, 5), but later improvements in the expression plasmid provided much larger quantities of intact Sak (6, 7). Some differences exist in the coding regions of the Sak genes in sakB$_C$, sak42D, and sakSTAR. Nonetheless, in each case, Sak is synthesized as a 163-amino acid protein that contains a 28-amino acid residue signal sequence. The x-ray crystallographic structure of SakSTAR has been determined, thus allowing a three-dimensional modeling of this protein (8).

Sak does not possess proteolytic activity. Thus, as with streptokinase (SK), the ability of Sak to convert hPg to hPm, a process that requires cleavage of the Arg$_{601}$Val$_{652}$ peptide bond in hPg (9), is indirect. However, unlike SK, Sak requires catalytic amounts of preformed hPm for this activation to occur (10–13), a feature that illustrates important differences in the mechanisms by which these two bacterial proteins activate hPg. Specifically, whereas the complex between SK and hPg is capable of self-generation of the enzymatic species SK-hPg and SK-hPm, both of which serve as hPg activators (for a review, see Ref. 14), the Sak-hPm complex must be processed by another hPg activator to provide Sak-hPm, which then functions as a hPg activator complex. During the conversion of hPg to hPm by Sak, a decapeptide is cleaved from the amino terminus of Sak (1). Although this peptide cleavage is not a rate-limiting step in hPg activation (15), it has been concluded that this event is necessary for this activation to occur (16).

Interest in Sak is based on its ability to enhance fibrinolysis in a fibrin-dependent manner, thus holding promise for Sak as an agent for thrombolytic therapy (17, 18). Therefore, elaboration of the essential properties of Sak required for hPg activation represents a timely area of investigation. In analysis of the amino acid sequence of SakSTAR, only one consensus site for N-linked glycosylation (Asn$_{28}$Val-Thr) is present. To test the importance of the occupancy of Asn$_{28}$ by carbohydrate, which becomes a relevant issue for the expression of SakSTAR in systems other than bacteria, we examined the ability of this locus to be processed in a yeast-derived expression system and the effects of glycosylation on the properties of this protein.

This communication presents the results of this study.
PCR was carried out using Pfu polymerase (5.0 units) and the pMEX602sakB plasmid (100 ng). The primer annealing temperature was 47 °C. The PCR products were purified by electroelution and cloned into pPIC9K via its AvrII and NolI restriction sites.

**Expression and Purification of Recombinant SakSTAR from E. coli**—DH5α cells containing the pMEX602 intracellular expression plasmid were grown in 100 ml of Terrific Broth (Sigma Chemical Co.) at 37 °C with vigorous shaking in 500-ml culture flasks. Induction was accomplished during the late log phase with 200 μM isopropyl-β-D-thio-β-galactopyranoside for 4–8 h. Cells were centrifuged, and pellets were resuspended in 0.25 the volume of culture medium in 0.04M sodium phosphate, pH 6.0, and disrupted by pulsed sonication for 5 min at 4 °C. Cell debris was separated by centrifugation at 45,000 × g for 30 min. The cleared lysates were diluted with H2O to 0.01 M sodium phosphate, pH 6.0. SakSTAR was then purified using minor modifications of a previously described method (7). First, the solution was purified by cation exchange chromatography over a 10-ml bed volume of SP-Sepharose at room temperature. The column was washed with 0.01 M sodium phosphate buffer, pH 6.0, and eluted with a 200-ml (total volume) linear gradient from 0 to 2.0 M NaCl. The pooled fractions containing SakSTAR, as identified by an S2251 chromogenic substrate assay, SDSPolyacrylamide gel electrophoresis, and TOF-MALDI-DE, were adjusted to 2.5 M NaCl and applied to a 10-ml bed volume of phenyl-Sepharose. The column was washed with 0.01 M sodium phosphate and 2.5 M NaCl, pH 6.0, and eluted with 0.01 M sodium phosphate, pH 6.0. Fractions containing SakSTAR were identified as described above.

**Expression and Purification of Recombinant SakSTAR from P. pastoris Cells**—The cDNA encoding SakSTAR was also expressed in the P. pastoris yeast system using the GS-115 strain. Detailed procedures used in this laboratory to select appropriate yeast transformants have been published previously (19). After transformation and selection of the high producing clones, the cells were grown to a cell density of 250 g/liter in a 5-liter fermentor using glycerol as the primary carbon source, after which methanol was added, and the cells were allowed to ferment for 8–12 h to induce extracellular production of SakSTAR. The medium was then collected and dialyzed against two changes of H2O to remove the high quantity of salt contained in the fermentor medium and, finally, against two changes of 0.01 M sodium phosphate, pH 6.0. SakSTAR was then purified in a two-step procedure as described above for the E. coli expression system. Fractions contained both SakSTARa and SakSTARa. To separate these uncleaved and glycosylated forms of the protein, the solution was dialyzed against 10 mM sodium phosphate, 5 mM MgCl2, and 400 mM NaCl, pH 7.0, and purified by affinity chromatography over a 10-ml bed volume of concanavalin A-Sepharose (Sigma Chemical Co.) at room temperature. SakSTAR was collected in the flow-through and wash. SakSTAR was then eluted from the column with 500 mM α-D-mannopyranoside in 10 mM sodium phosphate, 5 mM MgCl2, and 400 mM NaCl, pH 7.0.

When SakSTAR was to be used for carbohydrate analysis, elution from the concanavalin A-Sepharose column was accomplished using 1 M acetic acid to avoid any possible contamination with mannose. However, in this case, further purification was necessary. The SakSTAR preparation was dialyzed against H2O containing 0.1% trifluoroacetic acid and injected onto a 4.6 × 150-mm C8 reverse phase HPLC column (Vydac, Hesperia, CA) that was prequillibrated with a 75:25 (v:v) H2O/CH3CN solution containing 0.1% trifluoroacetic acid. Elution was accomplished by increasing the CH3CN to 75% over 40 min at a flow rate of 1 ml/min. The major peak that eluted at 19.5 min contained highly purified SakSTARa.

**Activation of hPg by SakSTAR**—To determine the plasminogen activator activity of SakSTAR, SakSTARa, and SakSTARa, the conversion of hPg to hPm was monitored using a coupled assay. An amount of 5 nm SakSTAR, SakSTARa, or SakSTARa was added to 5 nM hPg that contained endogenous trace amounts of hPm. The chromogenic substrate, a-D-mannopyranoside in 10 mM sodium phosphate buffer, pH 6.0, and disrupted by pulsed sonication for 5 min at 4 °C. The reaction mixture was incubated at 37 °C for 1 ha at 37 °C. The rate of activation of hPg by the SakSTAR variants was determined by the increase in absorbance at 405 nm as a function of time. The buffer used was 10 mM Na-Hepe and 150 mM NaOAc, pH 7.4.

To examine the activity of the prefolded SakSTARa-hPm complexes, the purified SakSTAR samples were added to hPm at a 1:1 molar stoichiometry. Urokinase was used to activate hPg before the addition of SakSTAR (0.5 mM urokinase and 13.5 μM hPm for 1 h at 37 °C). The activated hPm was then incubated with SakSTAR, SakSTARa, or SakSTARa at 37 °C to form the corresponding complex, respectively. Cytolytic amounts (1:30, m:m) of these enzyme complexes were then added to 25 nM hPg in activation buffer containing the substrate. The continued activation of hPg was monitored using a coupled assay, SDS-polyacrylamide gel electrophoresis, and TOF-MALDI-DE.

**Properties of N-Glycosylated Staphylokinase**
uous activation assay was carried out as indicated above. In separate experiments to determine the amido-lytic activities of these same complexes, substrate S2251 was used without the addition of hPg.

**Determination of Protein Concentrations and Spectrophotometric Absorbance by Analytical Ultracentrifugation—**SakSTAR samples were dialyzed against 10 mM sodium phosphate buffer, pH 6.0, and adjusted to an adsorption range of 0.75–1.2 at 280 nm. The absorption spectra and the interference fringe pattern were measured on a Beckman XL-I analytical ultracentrifuge with integrated absorbance and Rayleigh interference optics. A synthetic boundary cell was used with a path length of 1.2 cm. The volumes of the buffer reference and SakSTAR samples were 400 and 190 μl, respectively. Measurements were made at a rotor speed of 20,000 rpm at 20 °C with a detection wavelength of 280 nm for absorbance and 675 nm for fringe displacement. The fringe displacement (∆f) was determined by subtracting the mean value of the baseline from the plateau. The concentration (C) of the protein sample was then related to the fringe displacement by

\[ C = \frac{\Delta f}{d 
abla n/dc} \]

where \( \Delta f \) is the specific fringe displacement for a cell optical path length (L), defined as

\[ D = \frac{dn}{dc} \]

at the wavelength (λ) of the laser light source (675 nm), a value that is generally accepted to be independent of the amino acid composition of the protein.

**Differential Scanning Calorimetry—**Samples of SakSTAR, SakSTARu, or SakSTARg were dialyzed overnight against PBS buffer (0.01 M sodium phosphate and 0.14 M NaCl, pH 7.4). Protein concentrations of 0.75–1.3 mg/ml of SakSTAR, SakSTARu, or SakSTARg were determined at 280 nm using the experimentally determined molar extinction coefficient of 9,800. Protein samples and buffers were degassed for 30 min. All SakSTAR samples were heated at 1 °C/min, and the denaturation temperatures (Tm) and calorimetric enthalpies (H) for each protein were determined from computer analysis (with manufacturer-designed software) of the thermograms.

**Circular Dichroism (CD)—**CD spectra were recorded at 222 nm on an AVIV model 62DS spectrophotometer. SakSTAR, SakSTARu, or SakSTARg was dialyzed against PBS, pH 7.4, and concentrated to 0.2 mg/ml. A 0.2-μm path length cell was used. The data were collected at a 1.0-nm bandwidth, and each represents the average of three measurements. The SakSTAR samples were heated at 1 °C/min, and changes in ellipticity were monitored at 222 nm. Mean residue ellipticities were calculated using a mean residue molecular weight of 114.6 for SakSTAR (20). Points were fitted to a Boltzman curve, and midpoints were established as Tm values.

**Enzyme-linked Immunosorbent Assays—**A constant concentration of hPm (50 μl) was placed in individual wells of a 96-well microtiter plate, and the plates were washed with PBS buffer after an overnight incubation at 4 °C. A 2-h incubation with blocking buffer (6% milk in PBS buffer) was followed by washings with PBS buffer. SakSTAR, SakSTARu, or SakSTARg was then added to the wells at a series of concentrations between 0 and 100 ng/ml. After a 2-h incubation at room temperature, the wells were washed once again as described above. A mouse anti-SakSTAR IgG monoclonal antibody (5 μg/ml, obtained from H. E. Lijnens) was then incubated in the wells, and the wells were washed after 90 min. Alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA) was then added to the wells and allowed to incubate for 90 min. After washing, the substrate Sigma-104 (Sigma Chemical Co.) was added, and the absorbancies of the wells were determined at 405 nm in a microtiter plate reader to detect the amount of alkaline phosphatase present. After subtraction of the values determined for light scatter (absorbancy at 490 nm), plots were fitted to a hyperbolic curve, and C50 values were determined from the midpoints using Origin software. Control experiments demonstrated that the monoclonal antibody reacted with equal affinity to the three forms of SakSTAR.

**Sedimentation Equilibrium Experiments using Analytical Ultracentrifugation—**Sedimentation equilibrium experiments were performed on a Beckman XL-I analytical ultracentrifuge at speeds of 18,000, 20,000, and 25,000 rpm with protein concentrations of 0.33, 0.5, and 1.0 mg/ml. Before centrifugation, the protein solutions were equilibrated in 10 mM sodium phosphate and 100 mM NaCl, pH 7.5, at 20 °C. Dialysates were loaded into the reference chambers for all experiments. The apparent molecular weights and deviations from a single fit as functions of the radius from the center of rotation were determined using the Beckman XL-I software that accompanied the instrument.

**Analytical Techniques—**Oligonucleotides were synthesized using phosphoramidite-based methodology on the Beckman Oligo 1000M DNA synthesizer. For DNA sequencing, the dideoxy chain termination method (21) using the sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH) was used with the ALFexpress sequencer (Pharmacia Biotech, Piscataway, NJ). Fluorescence labeling was accomplished with Cy5-DATP. All reagents for this procedure were purchased from Pharmacia. The sequencing gel was run at 1500 V, 60 mA, and 25 W at 55 °C. The laser power was stabilized between 700 and 800. The sequence was processed using software supplied by Pharmacia.

The SakSTAR variants were identified in part based on their molecular weight by TOF-MALDI-DE on a Voyager-DE Spectrometer (PerSeptive Biosystems, Framington, MA). The samples were dialyzed against H2O to remove any residual salts, after which they were concentrated to 0.1–1.0 mg/ml. A volume of 0.5 μl of sample was added to 0.5 μl of 10 mg/ml sinapinic acid in a mixture of 50:50 (v/v) H2O:CH3CN containing 0.1% trifluoroacetic acid on a 100-well sample plate. The drops were air dried. The dried samples were irradiated with a N2 laser (337 nm; pulse time, 4 ns). Linear mode positive ionizations were used. Signal transients were recorded at a time resolution of 5 ns.

For determination of the molecular weights of SakSTAR variants in the stoichiometric complex with hPm, the SakSTAR preparation was incubated at a 1:1 (m/m) complex with hPm for 40 min in a buffer containing 10 mM sodium phosphate, 5 mM MgCl2, and 400 mM NaCl, pH 7.0. The reaction mixtures were then dialyzed overnight against H2O at 4 °C. The molecular weights of the SakSTAR samples in the presence and absence of hPm were determined by TOF-MALDI-DE in the positive ion mode as described above.
Oligosaccharides were released from SakSTAR, by digestion with PNGase F. The conditions of digestion were 1 unit PNGase F/200 µg SakSTAR, in a total volume of 50 µl of a buffer containing 100 mM sodium phosphate, pH 7.2, for 18 h at 37 °C. Hydrazinolysis was also employed using Oxford GlycoSystems (Rosedale, NY) Glycoprep 1000 for automated performance with 1 mg of SakSTAR in the cell. The released oligosaccharides were then labeled with 2-aminobenzamide (22, 23). Methodology used for characterization of the fluorescence-labeled oligosaccharides by HPLC and by gel filtration using the Oxford GlycoSystems RAM 2000 GlycoSequencer has been described previously (22).

RESULTS

Recombinant SakSTAR was purified from conditioned culture media obtained from an E. coli-based expression system using a combination of SP-Sepharose (Fig. 1A) and phenyl-Sepharose (Fig. 1B) chromatography in quantities of approximately 300–400 mg/liter cell culture. As determined by TOF-MALDI-DE, the final material possessed a molecular weight of 15498.9 (calculated molecular weight, 15494.6), and the amino-terminal amino acid sequence was found to be Ser-Ser-Ser-Phe-15498.9 (calculated molecular weight, 15494.6), and the amino-terminal amino acid sequence was found to be Ser-Ser-Ser-Phe-

A determination was made of the nature of the N-linked glycosylation at the lone consensus Asn site contained on Asn46 of SakSTAR. After release of the N-linked oligosaccharides by PNGase F, the glycan pool was fluorescence-labeled, and the components were identified in terms of their equivalent glucose units based on their elution positions from a calibrated BioGel P4 column (Fig. 2). The elution profile of the sample shows the presence of Man9GlcNAc2, Man10GlcNAc2, and Man11GlcNAc2 were present in approximately equal amounts and represented >80% of the oligosaccharides that were released from SakSTAR. When hydrazinolysis was used as the method for release of N-linked and O-linked saccharides, an elution profile very similar to that seen in Fig. 2 was obtained. Further treatment of the entire neutral oligosaccharide pool of Fig. 1 with jack bean α-mannosidase followed by rechromatography on the BioGel P4 column resulted in the identification of a single fraction of the Man(1,4)GlcNAc(1,4)GlcNAc core.

To assess the comparative properties of SakSTARu and SakSTARg, their thermal stabilities were determined. Differential scanning calorimetry analysis (Fig. 3) yielded Tm values of 71.7 °C for SakSTARu and 71.8 °C for SakSTARg with calorimetric enthalpies of 80.3 and 84.8 kcal/mol, respectively. These are similar to a Tm value of 70.0 °C and an enthalpy of 83.1 kcal/mol obtained for SakSTAR (data not shown). Comparable Tm values for SakSTARu, SakSTARu', and SakSTARg were determined by the randomness of the residuals also provided in Fig. 4. The apparent molecular weights determined from this approach were 15,994 ± 214 for SakSTARu and 18,241 ± 347 for SakSTARg. These compare very closely with the calculated molecular weights for the monomeric species. Similar data were collected for bacterially expressed SakSTAR.

FIG. 3. Differential scanning calorimetric analysis of SakSTAR. Protein concentrations of 0.7 and 1.2 mg/ml of SakSTARu (A) and SakSTARg (B) in a buffer containing 10 mM sodium phosphate and 100 mM NaCl, pH 7.4, were heated at 1 °C/min. The temperatures of maximum heat capacity (Tm) for each protein were determined using the software accompanying the calorimeter.

The overall abilities of the various forms of SakSTAR to activate hPg were assessed in an assay wherein the various forms of SakSTAR were added to hPg in a continuous assay of plasmin formation. Formation of the SakSTAR-hPm activator complex occurred due to the presence of small amounts of hPm that are present in the hPg preparations. Under identical activation conditions, the results illustrated in Fig. 5A demonstrated that whereas SakSTARu displayed a high level of hPg activator activity under these conditions, SakSTARg did not generate an effective hPg activator complex with hPm. On the other hand, the data in Fig. 5B demonstrate that the addition...
of equimolar levels to preformed hPm did not inhibit the amidolytic activity of hPm toward S2251. However, Fig. 5C shows that the ability of a preformed SakSTAR, hPm complex to activate hPg is significantly diminished.

The ability of the SakSTAR proteins to associate with hPm was determined from the enzyme-linked immunosorbent assay-based binding experiments shown in Fig. 6. The data demonstrate that all three forms of the protein interact in a nearly identical manner to hPm, with C_{50} values of 16, 21, and 22 nM for SakSTAR, SakSTAR, u, and SakSTAR, g, respectively. These experiments were performed by titration of hPm with the SakSTAR variants. When similar titrations of insolubilized SakSTAR preparations were conducted with hPm (the complex was detected with a hPg-derived monoclonal antibody), the C_{50} values were nearly the same (−1.5-fold higher in each case) as those reported above.

Lastly, because amino-terminal proteolysis (at Lys^{10}\text{-}Lys^{11}) of Sak has been shown to be necessary for hPg activation to occur (3, 6), the amino-terminal amino acid sequences of these forms of SakSTAR were determined after complexation with hPm. After incubation of equimolar amounts of each form of SakSTAR with hPm for 20 min at 37 °C, the samples were subjected to SDS gel electrophoresis under reducing conditions. The band containing the relevant SakSTAR was excised, and the amino-terminal amino acid sequence analysis was performed. For all three samples, the sequence (>90%) obtained was Lys^{1}\text{-}Gly-Asp-Asp-Ala-Ser. Further confirmation that this critical cleavage of SakSTAR occurs in the SakSTAR-hPm complex was achieved using TOF-MALDI-DE analysis. The SakSTAR, u, and SakSTAR, g, complexes displayed molecular mass differences of 1164 and 1203, respectively, from their parent proteins in the presence of hPm. These values (or their Na\textsuperscript{+} adducts) are consistent with the loss of a decameric peptide from the amino terminus of SakSTAR (calculated molecular weight, 1170.6).

**DISCUSSION**

SakSTAR contains a single N-linked oligosaccharide consensus site at Asn^{29}. This investigation was initiated to ascertain the effects on the activity of this protein consequent to glycan occupancy of that site. In its natural state in S. aureus, the relevant Asn^{29} is not glycosylated, but in expression systems other than bacteria, this residue is potentially capable of assembling oligosaccharide. A full understanding of whether this residue can be modified in the conformational environment of the intact protein and how processing at this site in other host cells affects its properties is relevant to the elucidation of the structure-function relationships of SakSTAR. An additional consideration concerns the extent to which SakSTAR activity is altered by glycosylation in expression systems that could be effectively utilized for its preparation. This is especially pertinent if large amounts of material would be required for use as a therapeutic fibrinolytic agent.

We used yeast cells of the strain P. pastoris as an expression system to express SakSTAR. The choice of this expression vehicle was governed by the potentially large amounts of secreted protein that could be obtained in a simple and inexpensive culture medium under simple fermentation conditions with methanol/glycerol as the only carbon sources. Furthermore, when compared with the much more extensively characterized Saccharomyces cerevisiae system, the glycosylation machinery of P. pastoris cells only permits assembly of relatively short N-linked high-mannose saccharides (22), a subpopulation of which may be phosphorylated (23). O-linked glycans with a small number of mannose residues consisting of mannose and (α1,2)-linked dimer to pentamer saccharides of mannose have also been observed (24).

**Fig. 4. Sedimentation equilibrium analysis of SakSTAR at 20 °C.** The concentration distribution of SakSTAR, u (top) and SakSTAR, g (bottom), measured as the absorbance at 275 or 235 nm, is shown as a function of radial distance from the center of rotation in the cell at equilibrium. The rotor speed was 32,000 rpm, and the temperature was 20 °C. Molecular weight (MW) was calculated as follows: MW = \( \frac{1}{L_0} \left( \frac{C_m - C_b}{C_m} \right) V(r^2 - r_m^2) \), \( C_m \) and \( C_b \), concentration of the protein at cell bottom and cell meniscus, respectively; \( r_m \), distance from the center of rotation to the cell bottom and cell meniscus, respectively; \( L_0 = \left( \frac{1}{v} \right) \text{spin/2πRT} \), where \( v \) represents the partial specific volume of the protein, \( v \) represents the solution density, and \( w \) represents angular velocity of the rotor. The buffer was 10 mM sodium phosphate and 100 mM NaCl, pH 7.4, at 20 °C. The calculated partial specific volume of SakSTAR, u was 0.747 ml/g. The same value was used for SakSTAR, g. The distributions of residuals for the indicated fits are shown in the top panels.

Purification of SakSTAR from 3 liters of fermentation medium of P. pastoris cells using an expression plasmid that allows for secretion of the protein results in total final yields of approximately 100–150 mg of the recombinant material. The relative amount of protein that contains N-linked glycans ranges from 50% to 75% of the total, which varies in different fermentations. We have not performed a systematic study of the routine variables in the fermentation protocol that affect the extent of protein modification.

The purified glycosylated and aglycosylated proteins were subjected to molecular weight analysis by TOF-MALDI-DE and amino-terminal acid sequence analysis. Both analyses indicated that signal peptide processing was identical for SakSTAR, u and SakSTAR, g, and yielded the same mature protein as that obtained for bacterially expressed SakSTAR. Neither SakSTAR, u nor SakSTAR, g displays association or concentration-dependent aggregation in solution as demonstrated by sedimentation equilibrium centrifugation. Thus, both yeast-expressed...
Fig. 5. Activation of hPg by SakSTAR. A. SakSTAR_u (Sak-u) or SakSTAR_g (Sak-g) (0.2 nM, final concentration) was added to hPg (5 nM, final concentration) containing a trace amount of hPm in the presence of 5 mM S2251. The conversion of hPg to hPm was monitored by the increase in absorbance at 405 nm due to the release of p-nitroanalide from the substrate by the hPm formed. B, hPg was activated with a catalytic level of urokinase. After full activation was accomplished, a quantity of SakSTAR_u (5 nM, final concentration) was added to the hPm (5 nM, final concentration), and the increase in absorbance at 405 nm in the presence of hPm (5 nM, final concentration) and SakSTAR_u/hPm (5 nM in hPm) was monitored with time. The buffer used was 50 mM Na-Hepes, 100 mM NaCl, and 1 mM CaCl_2, pH 7.4. C, plasminogen activator activity of SakSTAR_u/hPm complexes. Catalytic amounts (1 nM) of SakSTAR_u/hPm, SakSTAR_g/hPm, or hPm were added to 25 nM hPg containing substrate S2251 (0.9 mM). The conversion of hPg to hPm was monitored by the absorbance at 405 nm due to the release of p-nitroanalide from the substrate by the hPm formed. The buffer used was 10 mM Na-Hepes and 150 mM NaOAc, pH 7.4.
proteins exist as single chain molecules in solution.

The nature of the Asn28-linked oligosaccharides assembled on SakSTARg by P. pastoris cells was similar to that found earlier for another peptide expressed in this system (22). Man10GlcNAc2 and Man11GlcNAc2 represented approximately 80% of the total glycans released from Asn28. Exoglycosidase-catalyzed digestion of the entire oligosaccharide pool with an α-mannose-specific jack bean mannosidase showed that all mannose residues except the single core mannose possessed α-anomeric linkages. Digestion of this same oligosaccharide pool with (α1,2)-specific mannosidase yielded a single product, Man6GlcNAc2. This conclusion is consistent with previous results showing that the P. pastoris-derived core glycan structure contains an additional (α1,6)-mannose on the arm of the (α1,3)-mannose of the mammalian-type core oligosaccharide Man6GlcNAc2, with mannose extensions from this latter yeast-based core structure of the (α1,2)-type (22). Based on the HPLC profiles of N-linked oligosaccharides and on the lack of additional saccharide release by hydrazinolysis from SakSTARg after liberation of N-linked glycans by PNGase F, no evidence was obtained for charged N-linked oligosaccharides or O-linked saccharides, respectively, on SakSTARg. Thus, all carbohydrate chains assembled on this protein were Asn28-linked neutral high-mannose oligosaccharides with yeast-type mannose extensions from the mammalian-like core.

To assess the conformational stability of the protein, thermal stability measurements were carried out by two independent techniques. From both differential scanning calorimetry analysis and CD-monitored temperature scans, Tm values between 70 °C and 76 °C were obtained for SakSTAR, SakSTARu, and SakSTARg. These high Tm values suggest that the native conformation is highly resistant to thermal denaturation. This property is displayed despite the lack of added stability that would be present if the protein maintained disulfide bonds. Furthermore, the presence of the glycan does not significantly alter the conformational stability of SakSTAR. This conclusion also suggests that interactions between the protein and the oligosaccharide chain, if any, have minimal impact on the maintenance of stability in SakSTARg.

Measurements of the comparative abilities of the P. pastoris-derived SakSTAR preparations to activate hPg were made. When added to identical preparations of hPg that contained a small amount of hPm, it was found that whereas SakSTARa possessed the capacity to activate hPg, SakSTARg was nearly inactive under these conditions (Fig. 5A). This result then prompted additional experiments designed to address the basis for these differential effects. The first set of experiments was intended to address the question of whether the oligosaccharides present on SakSTARg could have precluded the formation of the SakSTAR-hPm activator complex. The data in Fig. 6 demonstrate that this is not the case, and complexes of nearly equal affinity were formed between hPm and each form of SakSTAR.

It has been found that release of a decapeptide from the amino terminus of SakSTAR, consequent to cleavage at Lys10-Lys16, is required for generation of an effective SakSTAR-hPm activator complex (3, 6). We then examined the possibility that the presence of the carbohydrate at Asn28 is affecting plasmin-mediated processing of SakSTAR to its fully active low molecular weight form. This was determined through incubation of both SakSTAR forms with hPm followed by electrophoretic separation. Amino-terminal sequence analysis of the excised gel band corresponding to SakSTARa showed a clear sequence, beginning at Lys10. This indicates that the requisite peptide bond in SakSTARa was cleaved in the complex, thus potentially rendering the complex capable of displaying hPg activation activity.

The possibility that the oligosaccharides present on SakSTARg could have blocked the active site of hPm, thus inhibiting the activity of the SakSTARg-hPm complex, was eliminated based on the experiments in Fig. 5B. Here, it is seen that the addition of an equimolar level of SakSTARg to hPm had little effect on the amidolytic activity of this enzyme toward the small substrate, S2251. However, upon activation of hPg with catalytic levels of preformed complexes of SakSTARg-hPm and SakSTARa-hPm (Fig. 6C), a clear diminution of SakSTARg-hPm activity, similar to that of hPm alone, is observed. Thus, we conclude that the basis of the poor hPg activator activity of SakSTARg results from the loss of specificity of the SakSTARg-hPm complex for hPg, perhaps due to a more restricted access of hPg to the active site of hPm in the activator complex.

Examination of a recently reported x-ray crystallographic structure of a ternary microplasmin-SakSTAR-microplasmin complex wherein an activating complex of SakSTAR and mi-
croplasmin is bound to a second substrate-like molecule of microplasmin (25) reveals that a Met residue at position 26 of SakSTAR is part of a hydrophobic network having surface complementarity to the carboxyl-terminal region of the microplasmin. This Met residue has been shown to be critical for the efficient activation of hPg by SakSTAR (26) and lies in close proximity to Asn28. The results displayed in Fig. 6 demonstrate that no appreciable decrease in hPm binding affinity is associated with glycosylation of SakSTAR. This observation strongly suggests that the presence of the oligosaccharide moiety on Asn28 is not interfering with the docking of the microplasmin moiety. However, a subtle change in orientation of hPm at the SakSTAR interface may be occurring, which can then restrict access of the activation loop of the hPg substrate to its requisite subsites in the active site cleft. The active site entry and proper orientation of a smaller substrate is not affected in the SakSTAR-hPm complex.

In conclusion, this investigation has provided clear evidence that glycosylation of the lone N-linked consensus site in SakSTAR is detrimental to its hPg activator activity and has revealed the mechanism of this effect. As such, this study provides valuable contributions to understanding the structure-function relationships of this protein. In addition, whereas oligosaccharides assembled on SakSTAR other than those identified herein may not produce these same effects, this work should serve to heighten awareness that processing events of this protein in other systems may not yield fully functional protein.

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Glycosylation of Asparagine-28 of Recombinant Staphylokinase with High-Mannose-type Oligosaccharides Results in a Protein with Highly Attenuated Plasminogen Activator Activity
Robert G. Miele, Mary Prorok, Vincent A. Costa and Francis J. Castellino

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