acknowledge an NIH traineeship in biotechnology (GM08346) to K.L.S. and the award of a Fannie and John Hertz Foundation Fellowship to K.W.R.

Supplementary Material Available: Synthetic details and spectroscopic data for **2a**, **2b**, **3** and **4**, including ROESY spectra and variable temperature data for **2a** and **4**, and enzyme kinetic data for all substrates analyzed (9 pages). Ordering information is given on any current masthead page.

A Mechanistic Proposal for Asparagine-Linked Glycosylation

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The origins of specificity and reactivity are presently unresolved issues in our understanding of co- and post-translational protein modification.[1] Central to unraveling the mechanism of asparagine-linked glycosylation[2] is ascertaining how the reactivity of a relatively poor nucleophile, the carboxamido group, might be enhanced. We have demonstrated[3] that glycosyl transfer acceptor properties may be related to the ability of the peptide to adopt an Asx-turn motif.[4] For glycosylation, the specific hydrogen-bonding interactions in this motif also bring the carboxamido oxygen of the asparagine side chain and the hydroxyl proton of the "essential" hydroxy amino acid into proximity. This added interaction may then provide additional stabilization for the Asx-turn and align the functional groups that comprise the specificity determinants for the process.

Mechanistic models to explain primary amide reactivity in the oligosaccharyltransferase-catalyzed (OT-catalyzed) process have previously been proposed by Marshall[5] and Bause et al.[6] While both models consider that the hydroxy amino acid functions by assisting ionization at nitrogen, neither model recognizes the potential importance of peptide backbone interactions nor implicates the Asx-turn in providing for a uniquely reactive side chain.

Toward deriving a mechanistic picture consistent with the observed conformational model, we have examined the glycosyl acceptor properties of peptides **1–5** in which the side chain functionality of the asparagine residue is replaced by groups with different ionization properties (Figure 1). The transfer kinetics of these peptides were determined[7] and compared against the standard substrate **1** (Table I). As a result of these studies, we propose that the enhanced asparagine reactivity may be attributed to a conformationally specific neighboring group assistance.

Asp-Xaa-Thr sites, such as **2**, are not glycosylated.[8,9] In addition, peptide **2** shows no competitive binding to OT, although the peptide adopts an Asx-turn conformation analogous to that observed in the substrates.[4] This suggests that a negative charge is not tolerated at the active site. Accordingly, those mechanisms which propose involvement of a nitrogen anion are unlikely, since **2** mimics that charge distribution and thus should inhibit OT as

(1) Yan, S. C. B.; Grinnell, B. W.; Wold, F. *Trends Biochem. Sci.* **1989**, *14*, 264.
(2) Kaplan, H. A.; Welply, J. K.; Lennarz, W. J. *Biochim. Biophys. Acta* **1987**, *906*, 161.
(3) Imperiali, B.; Shannon, K. L.; Rickert, K. W. *J. Am. Chem. Soc.*, preceding paper in this issue.
(4) Abbadi, A.; Mcharfi, M.; Aubry, A.; Premilat, S.; Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1991**, *113*, 2729.
(5) Marshall, R. D. *Ann. Rev. Biochem.* **1972**, *41*, 673.
(6) Bause, E.; Legler, G. *Biochem. J.* **1981**, *195*, 639.
(7) Imperiali, B.; Shannon, K. L. *Biochemistry* **1991**, *30*, 4374. Kinetic results are generated from a single crude microsomal preparation in order to circumvent inherent variations in the absolute activity.
(8) Gavel, Y.; von Heijne, G. *Protein Eng.* **1990**, *3*, 433.
(9) Bause, E. *FEBS Lett.* **1979**, *103*, 296.

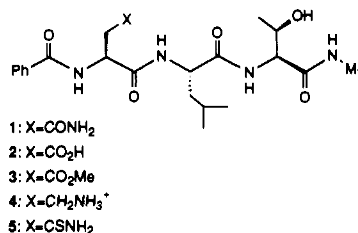

Figure 1.

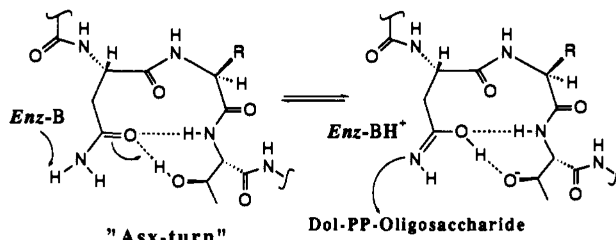

Figure 2.

Table I. Enzyme Kinetic Analysis of Tripeptides[a]

peptide	apparent K_M (mM)	rel V (%)[b]	K_i (mM)
Bz-Asn-Leu-Thr-NHMe (**1**)	0.24	100	
Bz-Asp-Leu-Thr-NHMe (**2**)			>10[c]
Bz-Asp(OγMe)-Leu-Thr-NHMe (**3**)			>10[c]
Bz-Amb-Leu-Thr-NHMe (**4**)			1.0
Bz-Asn(γS)-Leu-Thr-NHMe (**5**)	0.26	8.4	

[a] Determined in the presence of 200 000 dpm, 6.1 Ci/mmol $(GlcNAc)_2$-P-P-Dol (4×10^{-9} M).[7] [b] Peptide **1** as standard. [c] No inhibition was observed at concentrations below 5 mM.

a transition-state analog.[10] Likewise, **3** is not recognized by OT. This modification does not affect either the conformation of the peptide or the hydrogen-bonding interaction to the hydroxyl proton. However, the side chain no longer carries a proton donor, and this appears to impact binding. Peptide **4** competitively inhibits OT and has a K_i comparable to the K_M of the standard substrate **1**. This peptide lacks a carbonyl oxygen so it cannot form the necessary hydrogen-bonding interactions to stabilize the Asx-turn; however, it can achieve an analogous structure. Finally, the thioasparagine-containing[11] peptide, **5**, is a substrate for OT with a K_M similar to that for **1**.

Therefore, the important criteria to be considered in formulating a working hypothesis for the catalytic mechanism of OT are the following: the role of the Asx-turn, the lack of evidence for an intermediate bearing a formal negative charge at nitrogen, and the effect of differing proton donors at the carboxamide site.

A mechanism consistent with these considerations is shown in Figure 2. It is proposed that the peptide backbone interactions and the essential hydroxy amino acid provide the necessary machinery for protonation of the carboxamido oxygen and, simultaneously, a general base at the active site abstracts a proton from the nitrogen forming the imidate tautomer, which is a competent nucleophile. It is intriguing to note that the Asx-turn interaction juxtaposes the critical hydroxyl group with the syn lone pair of electrons on the carboxamido oxygen.[12] Rate acceleration may be affected either by catalysis of a rate-determining tautomerization or by stabilization of the imidate tautomer in the environment created by the peptide. Modifications which affect either the pK_a of the nitrogen protons or the basicity at the oxygen site should influence the binding to OT.

(10) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271.
(11) The steric and electronic implications of this substitution have been discussed by Sherman and Spatola: Sherman, D. B.; Spatola, A. F. *J. Am. Chem. Soc.* **1990**, *112*, 433.
(12) As suggested by studies on the carboxyl functional group (Gandour, R. D. *Bioorg. Chem.* **1981**, *10*, 169), the precise directionality of the proposed protonation step may be important for allowing the tautomerization to pick the path of minimum resistance.

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Both **4** and **5** have lower pK_a values than **1**.[13] Since the K_i of **4** is similar to the K_M of **1**, the increase in acidity apparently compensates for the lack of stabilized Asx-turn structure. Reaction of peptide **4** to afford a product which is labile to the reaction conditions was considered; however, increased background hydrolysis of the lipid-linked donor is not observed. The turnover of **5** is significantly lower than that for **1**, which may reflect the diminished basicity of sulfur. This suggests that the subtle balance between the protonation and deprotonation steps is essential for efficient catalysis.

Thus, we propose that the primary sequence requirements establish neighboring-group assistance for the tautomerization process.[14] Consequently, the conformation of the peptidyl acceptor appears to establish a microenvironment surrounding the carboxamido group which may govern the exceptional specificity observed in asparagine-linked glycosylation.

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Supplementary Material Available: Synthetic details and spectroscopic data for **4** and **5** and enzyme kinetic data for all peptides analyzed (11 pages). Ordering information is given on any current masthead page.

(13) For comparison, the following pK_a values are pertinent: CH_3CONH_2, 15.1 (Bordwell, F. G. *Acc. Chem. Res.* **1988**, *21*, 456); $CH_3CH_2NH_3^+$, 10.63 (*Lange's Handbook of Chemistry*, 13th ed.; Dean, J. A., Ed.; McGraw-Hill: New York, 1985); CH_3CSNH_2, 13.4 (Walter, W.; Becker, R. F. *Justus Liebigs Ann. Chem.* **1969**, *727*, 71.).

(14) The potential importance of neighboring-group assistance has been discussed for preservation of the cis-enediol intermediate and the mechanism of the aldo–keto isomerases; Rose, I. A. In *The Enzymes*; Boyer, P. D., Krebs, E. G., Eds.; Academic Press: New York, 1972; Vol. 2, p 293.

Intramolecular Fluorescence Enhancement: A Continuous Assay of Ras Farnesyl:Protein Transferase

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Posttranslational addition of hydrophobic moieties (fatty acid acylation and prenylation) is a functionally essential modification for many proteins involved in intracellular signaling pathways.[1] Study of the transferase enzymes that catalyze these modifications is complicated by troublesome single-point methods for assaying their activity. We have developed a continuous fluorimetric assay for one of these enzymes, farnesyl:protein transferase (FPTase), that takes advantage of a common feature among all hydrophobic modification reactions: the increase in hydrophobicity about the reaction center of the acceptor substrate that occurs following conversion of substrate to product.

FPTase catalyzes the transfer of a hydrophobic farnesyl group (C_{15}) from farnesyl diphosphate (FPP) to a specific C-terminal cysteine residue of a protein substrate, forming a thioether bond and displacing inorganic diphosphate in the process[2] (Scheme I). Short peptides (≥4 residues) containing a C-terminal consensus recognition sequence (CXXX) can also serve as farnesylation acceptor substrates.[2b,c,3] Interest in FPTase has intensified because farnesylation is required for membrane association and biological function of *ras*-encoded proteins,[4] mutant forms of which play a biological role in over 20% of all human cancers and in greater than 50% of pancreatic and colon tumors.[5] Inhibition of FPTase represents a possible method for preventing relocation of mutant Ras from the cytosol to the membrane, thereby blocking its cell-transforming function. Since cytosolic mutant forms of Ras act as dominant negative inhibitors of the Ras signaling pathway, inhibition of FPTase activity would also lead to the in situ accumulation of an inhibitor specific both for the Ras pathway and for cancer cells.[6] Enzymological studies and the search for potent, specific inhibitors led us to consider alternatives to the existing single-point assays for FPTase activity, which are labor-intensive and generate radioactive waste.[7]

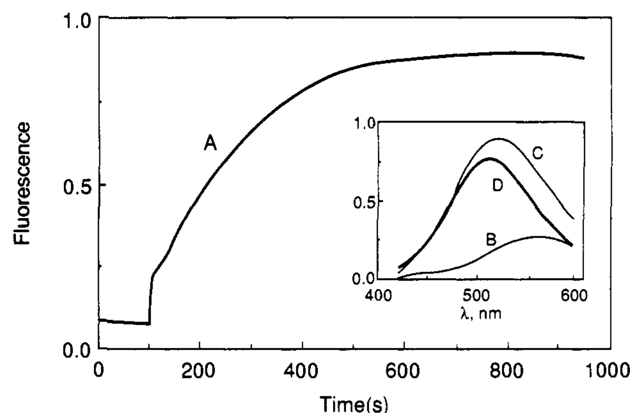
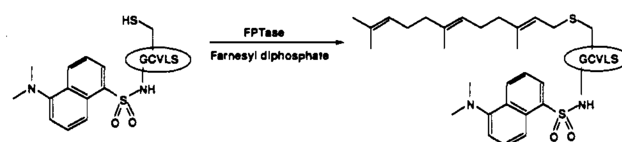


Figure 1. Fluorescence progress curve, A, of the reaction catalyzed by recombinant human farnesyl:protein transferase (0.5 nM) using Ds-GCVLS (1.0 μM) as substrate in the presence of a saturating concentration of farnesyl diphosphate (10 μM) in assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, and 0.2% octyl β-D-glucopyranoside) at 30 °C. Fluorescence data in the integration mode were obtained on a SPEX Fluorolog Model F112XI spectrofluorimeter with λ_{ex} = 340 nm (slit width = 4 nm) and λ_{em} = 505 nm (slit width = 8 nm) using 4 mm square microcells. Concentrations of stock solutions of Ds-GCVLS (in 20 mM Tris-HCl, pH 7.5, 10 mM EDTA) were calculated from the extinction coefficient of the dansyl moiety at 340 nm (ε = 4250 M^{-1} cm^{-1}). Inset: Fluorescence emission spectrum (λ_{ex} = 340 nm) of B, substrate Ds-GCVLS (1.0 μM in assay buffer); C, product Ds-G[f-C]VLS (1.0 μM in assay buffer); and D, difference between substrate and product.

Scheme I



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† Department of Cancer Research.
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(1) (a) James, G.; Olsen, E. N. *Biochemistry* **1990**, *29*, 2623–2634. (b) McIlhinney, R. A. J. *Trends Biochem. Sci.* **1990**, *15*, 387–391. (c) Gordon, J. I.; Duronio, R. J.; Rudnick, D. A.; Adams, S. P.; Gokel, G. W. *J. Biol. Chem.* **1991**, *266*, 8647–8650. (d) Glomset, J. A.; Gelb, M. H.; Farnsworth, C. C. *Trends Biochem. Sci.* **1990**, *15*, 139–142. (e) Maltese, W. A. *FASEB J.* **1990**, *4*, 3319–3328. (f) Rine, J.; Kim, S.-H. *New Biologist* **1990**, *2*, 219–226. (g) Der, C. J.; Cox, A. D. *Cancer Cells* **1991**, *3*, 331–340. (h) Sinensky, M.; Lutz, R. J. *BioEssays* **1992**, *14*, 25–31.

(2) (a) Manne, V.; Roberts, D.; Tobin, A.; O'Rourke, E.; Virgilio, M. D.; Meyers, C.; Ahmed, N.; Kurtz, B.; Resh, M.; Kung, H. F.; Barbacid, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7541–7545. (b) Schaber, M. D.; O'Hara, M. B.; Garsky, V. M.; Mosser, S. D.; Bergstrom, J. D.; Moores, S. L.; Marshall, M. S.; Friedman, P. A.; Dixon, R. A. F.; Gibbs, J. B. *J. Biol. Chem.* **1990**, *265*, 14701–14704. (c) Reiss, Y.; Goldstein, J. L.; Seabra, M. C.; Casey, P. J.; Brown, M. S. *Cell* **1990**, *62*, 81–88.

(3) Moores, S. L.; Schaber, M. D.; Mosser, S. D.; Rands, E.; O'Hara, M. B.; Garsky, V. M.; Marshall, M. S.; Pompliano, D. L.; Gibbs, J. B. *J. Biol. Chem.* **1991**, *266*, 14603–14610.

(4) (a) Willumsen, B. M.; Norris, K.; Papageorge, A. G.; Hubbert, N. L.; Lowry, D. R. *EMBO J.* **1984**, *3*, 2581–2585. (b) Hancock, J. F.; Magee, A. I.; Childs, J. E.; Marshall, C. J. *Cell* **1989**, *57*, 1167–1177. (c) Jackson, J. H.; Cochrane, C. G.; Bourne, H. R.; Solski, P. A.; Buss, J. E.; Der, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3042–3046.

(5) Bos, J. L. *Molecular Genetics in Cancer Diagnosis*; Cossman, J., Ed.; Elsevier Science Publishing Co.: New York, 1991; pp 273–288.

(6) Gibbs, J. B. *Cell* **1991**, *65*, 1–4.

0002-7863/92/1514-7945$03.00/0 © 1992 American Chemical Society