Enzymic O-Glycosylation of Synthetic Peptides from Sequences in Basic Myelin Protein†

Janis Dillaha Young,* Dorinne Tsuchiya,‡ Diane E. Sandlin,§ and Michael J. Holroyde¶

ABSTRACT: Nine synthetic peptides containing sequences in the region of a threonine residue at position 98 of bovine basic myelin protein were prepared by the Merrifield solid-phase method and tested for their ability to be glycosylated with [^{14}C]uridinediphospho-*N*-acetylgalactosamine and a crude detergent-solubilized preparation of uridinediphospho-*N*-acetylgalactosamine:mucin polypeptide *N*-acetylgalactosaminyltransferase obtained from porcine submaxillary glands. The tetrapeptide Thr-Pro-Pro-Pro and all larger peptides containing this sequence were glycosylated. The glycosylation was greater for peptides containing residues N-terminal to the Thr-Pro-Pro-Pro. Under the conditions used, the peptide Val-Thr-Pro-Arg-Thr-Pro-Pro-Pro was glycoslyated twice as much as bovine basic myelin protein. Thr-Pro and Thr-Pro-Pro, as well as 10 other synthetic peptides which did not contain the Thr-Pro-Pro-Pro sequence, were not glycosylated. Treatment of the glycopeptide of Phe-Lys-Asn-Leu-Val-Thr-Pro-Arg-Thr-Pro-Pro-Pro-Ser with an α-*N*-acetylgalactosaminidase released *N*-acetylgalactosamine from the peptide, indicating that the hexosamine was covalently bonded to the peptide in an α linkage.

The basic protein of myelin sheath comprises 30% of the protein of central nervous system myelin. When it is injected into experimental animals, it elicits an immunological demyelinating disease, experimental allergic encephalomyelitis (EAE). The function of this protein in the myelin sheath is unknown, but it may be important in retaining the structural integrity of the myelin sheath and in the transfer of materials across the sheath (Morell, 1977; Carnegie, 1971; Eylar et al., 1974; Smith, 1977). Three kinds of modifications of this protein occur in close proximity to each other. They are glycosylation at residue 98 (Hagopian et al., 1971), methylation of the guanidino group of arginine at 107 (Baldwin & Carnegie, 1971; Brostoff & Eylar, 1971), and phosphorylation at residues 98 and 110 (Daile et al., 1975; Chou et al., 1976). The phosphorylation and methylation of the protein have already been shown to occur in vivo. We are interested in determining if any of these modifications are important to the function of the protein and have undertaken a study of these reactions using peptides in place of the whole protein as substrates. We previously synthesized a region of the protein Gly-Arg-Gly-Leu-*Ser*-Leu-Ser-Arg (residues 106–113) and found that serine-110 can serve as a substrate in a cAMP-dependent protein kinase phosphorylation reaction (Daile et al., 1975). In this paper we present evidence that peptides with the sequence in the region of threonine-98 can be glycosylated with UDP-GalNAc[1] and a crude detergent-solubilized enzyme preparation obtained from porcine submaxillary glands (Schwyzer & Hill, 1977). These peptide substrates provide useful models for study of the O-glycosylation reaction, an extremely important reaction in many secretory proteins (Kornfeld & Kornfeld, 1976; Uy & Wold, 1977).

Methods

Peptide Synthesis. The peptides in Table I were synthesized by the solid-phase method of Merrifield as described by Stewart & Young (1969). Amino acid derivatives were all the L configuration and were purchased from Bachem (Torrance, CA), Fox Chemical (now Fox-Vega, Tucson, AZ), and Peninsula Laboratories (San Carlos, CA). The following derivatives were used: Boc-Arg(Tos), Boc-Asn(Xan), Boc-Gly, Boc-Leu, Boc-Lys(ClZ), Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), and Boc-Val. The xanthydryl derivative of asparagine was synthesized from Boc-Asn and xanthydrol as described by J. M. Stewart (personal communication) or purchased from Bachem. The following steps were used for coupling each residue: (1) wash with dichloromethane, 6 times; (2) deprotect with trifluoroacetic acid–dichlororomethane (1:3 v/v), containing 1 mg/mL indole, 10 min; (3) deprotect with same reagent, 20 min; (4) wash with dichloromethane, 6 times; (5) neutralize with triethylamine–dichloromethane (1:9 v/v), 1 min; (6) neutralize with same reagent, 9 min; (7) wash with dichloromethane, 6 times; (8) equilibrate with 2.5 equiv of Boc amino acid (dimethylformamide used as needed for solubility), 10 min; (9) add 2.5 equiv of dicyclohexylcarbodiimide and couple for 2 h; and (10) wash with dichloromethane, 6 times. Coupling reactions were monitored for completeness with the ninhydrin test (Kaiser et al., 1970). When the reaction was incomplete, the residue was recoupled by repetition of steps 4–10 above.

Peptide–resins were cleaved with anhydrous HF (10 mL/g of peptide–resin) containing 10% anisole for 30 min at 0 °C by the standard procedure (Stewart & Young, 1969). Peptides 1–7 were used without further purification. Peptides 8 and 9 were purified by preparative high-voltage electrophoresis at pH 3.5. Amino acid analyses were performed on a 120B Beckman analyzer following hydrolysis in sealed evacuated

† From the Department of Bacteriology and Immunology and the Department of Molecular Biology, University of California, Berkeley, California 94720. *Received November 8, 1978.* Supported by grants from the Kroc Foundation, National Institutes of Health Grant ROI NS 12548, and National Aeronautics and Space Administration Grant NG-05-003-460. A preliminary publication has appeared (Young et al., 1978).

* Present address: McLean Hospital, Belmont, MA 02178.

‡ Peninsula Laboratories, Inc., San Carlos, CA 94070.

§ Biochemistry Department, Duke University Medical School, Durham, NC 27710. Present address: Department of Physiology, University of Cincinnati Medical College, Cincinnati, OH 45267.

¶ Present address: Department of Plant Pathology, University of California, Berkeley, CA 94720.

[1] Abbreviations used: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; ClZ, 2-chlorobenzyloxycarbonyl; GalNAc, *N*-acetylgalactosamine; Tos, *p*-toluenesulfonyl; UDP-GalNAc, uridinediphospho-*N*-acetylgalactosamine; Xan, xanthydryl.

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tubes for 22 h at 110 °C in 6 N HCl. TLC on silica gel was done in solvents I, N, O, and F (Stewart & Young, 1969). High-voltage electrophoresis was performed in Savant tanks using Whatman 3MM paper for 1–3 h at 1150–3000 V. The buffers were the following: pH 3.5, acetic acid–pyridine–water (10:1:90); pH 5.7, pyridine–HOAc–H_2O (50:2:510); and pH 1.9, formic acid (88–90%)–HOAc–H_2O (452:150:806).

Preparation of the Detergent-Solubilized Enzyme. A crude Triton X-100 extract of UDP-GalNAc:mucin polypeptide *N*-acetylgalactosaminyltransferase was prepared from porcine submaxillary glands (Pelfreeze, Rogers, AK) by a modification of the procedure of Schwyzer & Hill (1977). Buffer B was 0.025 M imidazole–HCl, pH 7.5, containing 0.02 M $MnCl_2$, 0.2 M NaCl, and 0.2% Triton X-100, and the final concentration of Triton X-100 was 0.2% instead of 1%. The mixture was stirred for 1 h after the 0.2% Triton X-100 concentration was reached. The Triton X-100 extract was centrifuged for 1 h at 39000 rpm (3 °C), and the clear supernatant was transferred into 0.5-mL plastic beakers and kept at −60 °C. The enzyme was thawed only one time prior to use. The enzyme preparation contained 12 microunits/10 μL when assayed by the standard procedure of Hill et al. (1977b).

Glycosylation of Substrates by the Detergent-Solubilized Enzyme Preparation. The glycosylation reaction was performed essentially as described by Hill et al. (1977b) using Dowex 1-X8 chloride chromatography and high-voltage electrophoresis to identify and quantitate the products. The buffer was 0.4 M Tris, pH 7.0, containing 0.1% Triton X-100 and 0.08 M $MnCl_2$. The pH must be adjusted to 7 before adding the $MnCl_2$. The usual incubation mixture, 60 μL, contained the following: buffer, 25 μL; UDP-[^{14}C]GalNAc [New England Nuclear, lot 809-035, 47 mCi/mmol in ethanol–water (1:1)], 1 nmol, 5 μL; peptide or protein, 10 μmol/mL, dissolved in water and the pH adjusted to 7.0 with 0.2 M $NaHCO_3$, 10 μL; and the detergent-solubilized enzyme preparation, 20 μL. After incubation at 37 °C for the desired time in capped 0.5-mL conical plastic beakers, the reaction was stopped by the addition of 20 μL of 0.15 M EDTA. The reaction product was passed through a 0.4 × 4 cm column of Dowex 1-X8 chloride resin and eluted with four 0.2-mL portions of water into scintillation vials. The entire sample was either dissolved in 10 mL of Scintisol (Isolab, Akron, OH) and the radioactivity measured or an aliquot was removed for radioactive measurement and the remaining eluate concentrated and subjected to paper electrophoresis. After electrophoresis, the peptides were located with ninhydrin and the apomucin was located by the Sakaguchi stain (Stewart & Young, 1969). Radioactive spots were located by overnight autoradiography on X-ray film (Kodak no-screen film, ready-pack NS-2T). Background was reduced in the autoradiogram by covering the back emulsion with self-adhesive waterproof paper (CON-TACT) during development, and the CON-TACT paper was removed before fixing. Radioactivity was measured in a Mark I Nuclear Chicago liquid scintillation system.

α-N-Acetylgalactosaminidase Treatment of [^{14}C]GalNAc–Peptide 9. [^{14}C]GalNAc–peptide 9 (see Table I), 48 nmol, was eluted from electrophoretograms and the eluate dried in vacuo. The peptide was dissolved in 100 μL of 0.01 M sodium acetate buffer, pH 4, and divided into two 50-μL portions. To one portion we added 7 milliunits of limpet α-*N*-acetylgalactosaminidase (prepared in the laboratory of R. L. Hill), and both solutions were incubated for 42 h at 37 °C. After incubation, the solutions were lyophilized. The dried samples were dissolved in water and subjected to electrophoresis at pH 3.5. The electrophoretogram was exposed to X-ray film overnight and stained with ninhydrin.

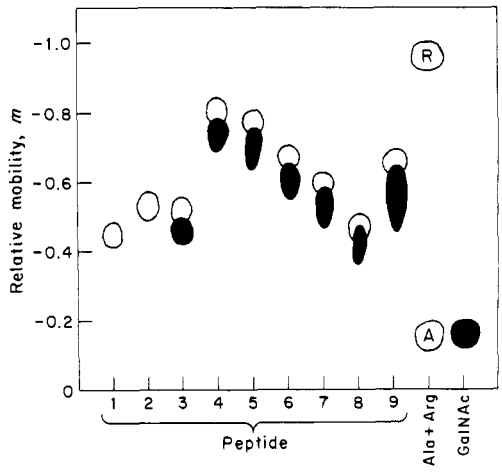

FIGURE 1: Relative mobility values (*m*) at pH 3.5 of reference compounds, [^{14}C]GalNAc–peptides 1–9, and unglycosylated peptides 1–9 (Table I). Radioactive areas are shaded, and ninhydrin-positive areas are unshaded.

Results

Characterization of Synthetic Peptides. Structures and analytical data on the nine synthetic peptides prepared for this study are given in Table I. Electrophoretic and TLC data on the peptides are given in Tables II and III. The peptides were essentially homogeneous after cleavage from the resin, as judged by their behavior on TLC and electrophoresis.

Glycosylation of Peptides. Each of the peptides in Table I was tested for its ability to serve as a substrate in the UDP-GalNAc:mucin polypeptide *N*-acetylgalactosaminyltransferase reaction. Figure 1 is a composite drawing of an electrophoretogram of the glycosylated and nonglycosylated peptides. Peptide 3 and all larger peptides were glycosylated. No glycosylation was obtained in the presence of EDTA or at a pH less than 6. The detergent-solubilized enzyme preparation contained peptidase activity. There were several radioactive spots of slower mobility on the electrophoretograms in addition to a major radioactive area migrating just behind the nonglycosylated peptide. There were ninhydrin-positive spots in addition to the nonglycosylated peptide upon incubation of the peptides with the enzyme preparation either with or without UDP-GalNAc. A comparison of the amount of glycosylation of peptides 1–7 (Table I) and myelin basic protein is given in Table IV. Under the conditions of the experiment, peptide 3 was a poor acceptor for GalNAc and peptides 4–7 were glycosylated as well as or better than the myelin basic protein.

Deglycosylation of N-Acetylgalactosaminyl–Peptide 9 by α-N-Acetylgalactosaminidase. The electrophoretic behavior at pH 3.5 of [^{14}C]-*N*-acetylgalactosaminyl–peptide 9 with and without α-*N*-acetylgalactosaminidase treatment is shown in Figure 2. The α-*N*-acetylgalactosaminidase-treated peptide gave one radioactive area coinciding with *N*-acetylgalactosamine. The nonenzyme-treated peptide had several minor radioactive areas, but none were in the area of *N*-acetylgalactosamine.

Peptides Not Glycosylated. The 10 synthetic peptides given in Table V were tested for their ability to be glycosylated. The peptides (0.1 mg) were incubated for 1 and 18 h with the transferase enzyme. Peptide 7 (Table I) was included in the same experiment as a control and was glycosylated, whereas none of the other peptides were glycosylated. Each peptide

Table I: Data on Synthetic Peptides of Myelin Basic Protein Region Containing Threonine-98

peptide no.	residue no. in protein and amino acid sequence of synthetic peptide															M_r	μmol of peptide obtained	mole ratio of residues in synthetic peptide[a]							
	90	91	92	93	94	95	96	97	98	99	100	101	102					K	R	D	T	S	P	V	L	F
1									T	P						216	951				0.97 (1)		1.04 (1)			
2									T	P	P					313	107				1.01 (1)		1.99 (2)			
3									T	P	P	P				410	60				0.91 (1)		3.08 (3)			
4								R	T	P	P	P				566	70		1.01 (1)		0.97 (1)		3.01 (3)			
5							P	R	T	P	P	P				663	45		0.92 (1)		1.07 (1)		3.99 (4)			
6						T	P	R	T	P	P	P				764	27		0.98 (1)		2.03 (2)		3.99 (4)			
7					V	T	P	R	T	P	P	P				863	344		1.06 (1)		1.94 (2)		4.06 (4)	0.94 (1)		
8					V	T	P	R	T	P	P	P	S			950	64		0.95 (1)		1.88 (2)	1.0 (1)	4.01 (4)	1.13 (1)		
9	F	K	N	L[b]	V	T	P	R	T	P	P	P	S			1452	137	0.81 (1)	0.95 (1)	0.88 (1)	2.03 (2)	1.05 (1)	4.15 (4)	1.01 (1)	0.98 (1)	0.85 (1)

[a] The numbers in parentheses are the expected mole ratios. [b] The leucine at position 93 in the synthetic peptide is isoleucine in the protein sequence.

Table II: Electrophoretic Properties of Synthetic Peptides Given in Table I[a]

peptide no. or compd	basic protein residue no.	m pH 1.9	m pH 3.5	m pH 5.6
1	98-99	−0.62	−0.49	−0.19
2	98-100	−0.53	−0.56	−0.20
3	98-101	−0.46	−0.55	−0.19
4	97-101	−0.71	−0.85	−0.48
5	96-101	−0.64	−0.80	−0.45
6	95-101	−0.59	−0.70	−0.40
7	94-101	−0.53	−0.63	−0.39
8	94-102	−0.51	−0.50	−0.34
9	90-102	−0.55	−0.58	−0.45
Arg		−1.0	−1.0	−1.0
Lys			−1.06	
Ala			−0.17	
UDP-GalNAc			+0.67	
Asp			+0.23	

[a] Mobilities, m, were calculated with reference to arginine as −1.0; migration to the cathode is indicated by − and migration to the anode as +. See the text for composition of the buffers.

Table III: Thin-Layer Chromatography of Synthetic Peptides of Basic Myelin Protein[a]

peptide no. or compd	protein residue no.	R_f 1	R_f 2	R_f 3	R_f 4
1	98-99	0.21	0.89	0.90	0.46
2	98-100	0.02	0.90	0.85	0.42
3	98-101	0.12	0.93	0.83	0.39
4	97-101	0.05	0.97	0.82	0.37
5	96-101	0.17	0.95	0.78	0.32
6	95-101	0.03	0.99	0.79	0.30
7	94-101	0.05	1.00	0.82	0.38
8	94-102	0.03	1.00	0.83	0.37
9	90-102	0.02	1.00	0.84	0.46
Asp		0.21	1.00	0.95	0.36
Ala		0.29	0.95	0.93	0.43
Arg		0.08	0.91	0.88	0.38

[a] Solvents: (1) 1-butanol-acetic acid-water (4:1:1); (2) pyridine-acetic acid-water (50:30:15); (3) 2-butanone-acetic acid-water (10:30:25); and (4) 1-butanol-acetic acid-water-pyridine (15:3:12:10). Approximately 0.03 mg of peptide was chromatographed.

Table IV: Comparison of Glycosylation of Synthetic Peptides and Bovine Myelin Basic Protein[a]

peptide no.	substrate	M_r	[^{14}C]-GalNAc eluted thru Dowex column (cpm)[b]	[^{14}C]-GalNAc on substrate (cpm)	([^{14}C]GalNAc on substrate)/([^{14}C]GalNAc on basic protein)
1	TP	213	929	−316	no reaction
2	TPP	313	1 096	−149	no reaction
3	TPPP	410	2 647	1 402	0.1
4	RTPPP	566	18 491	17 246	1.1
5	PRTPPP	663	20 514	19 269	1.2
6	TPRTPPP	764	19 670	18 425	1.1
7	VTPRTPPP	863	42 312	41 067	2.5
8	bovine basic protein	18 400	17 486	16 241	1
9	none		1 245	0	

[a] The incubations were performed for 1 h at 37 °C as described in the text. [b] The efficiency of counting was 86%.

was degraded to some extent by the crude enzyme preparation, especially after overnight incubation, but some of each of the original peptides remained under conditions where peptide 7 was glycosylated.

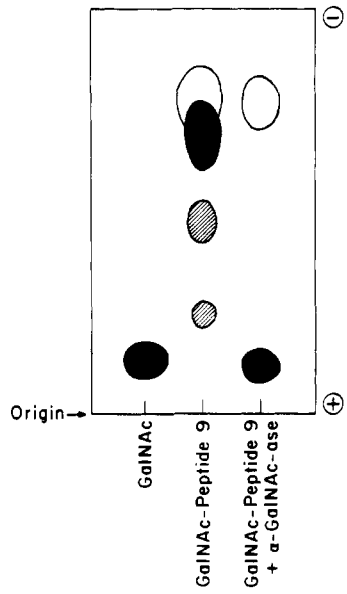

FIGURE 2: Electrophoretogram after electrophoresis at pH 3.5 of [^{14}C]GalNAc, [^{14}C]GalNAc–peptide 9 (Table I), and α-*N*-acetylgalactosaminidase (α-GalNAc-ase)-treated [^{14}C]GalNAc–peptide 9. Radioactive areas are shaded, and ninhydrin-positive areas are unshaded.

Table V: Synthetic Peptides Which Are Not Substrates for UDP-GalNAc:Mucin Polypeptide Transferase

peptide[a]	protein containing this sequence
TTASSF	prohistidine decarboxylase, hinge region
HADEGS	none known
acetyl-SYSME	MSH, residues 1–5
IRGERA	histone III, bovine, residues 53–57
TGMFNQ	TMVP, U-2, residues 136–141
RGTGMFNQ	TMVP, U-2, residues 134–141
LDATR	TMVP, vulgare, residues 108–112
TTAETLDATR	TMVP, vulgare, residues 103–112
QKSQRSQDENP	myelin basic protein, guinea pig, homologous to bovine residues beginning at 74
SRFSWGAEGQRPGFGYGG	myelin basic protein, bovine, residues 112–129

[a] The peptides were synthesized in our laboratory by the solid-phase method.

Discussion

Evidence is presented in this paper that low molecular weight synthetic peptides can be O-glycosylated with a crude preparation of UDP-GalNAc:mucin polypeptide *N*-acetylgalactosaminyltransferase obtained from porcine submaxillary glands. This finding was surprising from previous studies in which no small substrates were found for this enzyme. McGuire & Roseman (1967) first characterized this enzyme from ovine (sheep) glands, and they as well as Hagopian & Eylar (1968), using an enzyme from bovine glands, tested a number of proteins and small substrates with the combined result that only apomucin (deglycosylated submaxillary mucin) and basic myelin protein were substrates for the transferase. Hill et al. (1977b) more recently found that long peptides of 74 residues from ovine apomucin were easily glycosylated and shorter 16-residue peptides were poorly glycosylated by a porcine submaxillary transferase. In view of these observations, we prepared a relatively long peptide comprising residues 94–112 of bovine basic myelin protein for use in attempting to obtain a peptide substrate which could be glycosylated. This peptide and related 13- and 9-residue peptides (peptides 8 and 9, Table I) were all glycosylated (Young et al., 1978). We then synthesized the family of shorter peptides, peptides 1–7 (Table I), and found that peptide 3 contained the minimum sequence for glycosylation.

As illustrated in Figure 1, the [^{14}C]GalNAc–peptides 3–9 migrate on electrophoresis at pH 3.5 slightly slower than nonglycosylated peptides 3–9. This slower mobility of the glycosylated peptides was due to the larger molecular weight and the possible additional selective retardation due to the hydroxyl groups of the sugar moiety. The electrophoretic mobility indicates that the α-amino group of the peptide is free. The location of the sugar on peptide 3 is assumed to be on the hydroxyl group of the threonine, the only group present in the peptide which has been shown to be glycosylated in previous studies (Kornfeld & Kornfeld, 1976).

The [^{14}C]GalNAc–peptide 9 (Table I) area was eluted from electrophoretograms and subjected to the enzyme α-*N*-acetylgalactosaminidase. Reelectrophoresis at pH 3.5 of the α-*N*-acetylgalactosaminidase-digested peptide resulted in the disappearance of radioactivity in the area of GalNAc–peptide 9 and the appearance of a radioactive area where GalNAc migrates. The results of this experiment (see Figure 2) reveal that *N*-acetylgalactosamine is linked in an α configuration to peptide 9 and the sugar is not epimerized to another sugar. Hydrolysis of GalNAc-Thr-Pro-Pro-Pro released galactosamine, which was characterized on the amino acid analyzer (data not shown) by Dr. Roberta Palmour (University of California, Berkeley, CA).

Preliminary experiments have shown that *N*-acetylgalactosamine is released from several of the GalNAc–peptides via base-catalyzed β-elimination except for GalNAc–peptide 3 (Table I). This is not surprising since residues with free α-amino groups (Baenziger & Kornfeld, 1974; Derevitskaya et al., 1967) are resistant to β-elimination.

A comparison of glycosylation of the synthetic peptides 1–7 to bovine basic myelin protein is shown in Table IV. The glycosylation was better than the basic protein for peptide 7, V-T-P-R-T-P-P-P, and about the same as the basic protein for peptides 4–6. The smallest glycosylated peptide, T-P-P-P, was glycosylated to a much less extent than the longer peptides. In other experiments (data not given) the T-P-P-P was glycosylated to a greater extent, but was in all cases glycosylated less than the longer peptides. Preliminary experiments indicate that the T-P-P-P cannot be glycosylated at a pH below 7.0. The T-P and T-P-P were not glycosylated when incubated for 3 h, using 350 nmol of peptide and all other conditions the same as those in Table IV. We do not know whether one or two of the two threonines in peptides 6 and 7 are glycosylated. The work of Hagopian et al. (1971) strongly indicates that threonine-98 was the only residue glycosylated in the intact protein, but the steric configuration of this region in the intact protein may be different from that in the peptide.

A major problem in this work was that the crude porcine enzyme preparation contained protease activity. This proteolysis occurred with peptides but did not occur with apomucin or several other proteins. We were able to separate the proteolytic activity from the UDP-GalNAc transferase activity on a SP-Sephadex C-50 column (Hill et al., 1977b), but the transferase thus obtained was not stable. The enzyme has been isolated from the submaxillary glands of bovine, porcine, and ovine sources by different investigators. It is possible that the bovine enzyme which was previously obtained by Hagopian & Eylar (1969) contains less of the proteolytic activity and can be isolated as a more stable preparation. Hagopian & Eylar (1969) obtained the enzyme from freshly excised tissue, whereas our preparation was from frozen tissue.

The fact that peptides 1 and 2 (Table IV) as well as nine other serine- and threonine-containing peptides (Table V) were not glycosylated suggests that the transferase requires a high degree of substrate specificity. The failure of the peptides in Table V to serve as substrates was not an artifact of the peptide degradation by the protease activity in the transferase preparation since much of the original peptide remained during the time needed to glycosylate peptide 7 (Tables I and IV). No small peptide without a triproline sequence C-terminal to a threonine has been glycosylated by the enzyme in this work. This suggests that the three prolines C-terminal to the threonine provide a unique and suitable shape for the glycosylation. Indeed, the minimum structural requirement for O-glycosylation is contained in the tetrapeptide Thr-Pro-Pro-Pro.

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