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Enzymatic Conversion of Proteins to Glycoproteins By Lipid-Linked Saccharides: A Study of Potential Exogenous Acceptor Proteins

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Previous studies have shown that a membrane preparation from hen oviduct catalyzes transfer of oligosaccharide from oligosaccharide-P-P-dolichol to denatured RNase and α-lactalbumin. To gain further insight into the structural requirements of a protein that allow it to serve as a substrate for glycosylation, the acceptor ability of a variety of other modified proteins containing the tripeptide sequence -ASN-X-(SER/THR)- has been investigated. Of 7 proteins tested, 2 (ovine prolactin and rabbit muscle triosephosphate isomerase) could be enzymatically glycosylated by a particulate preparation from hen oviduct. The remaining 5 proteins, assayed as either S-carboxymethylated or S-aminoethylated derivatives, were inactive as carbohydrate acceptors. However, cyanogen bromide treatment of 2 of the inactive proteins, bovine catalase and concanavalin A from jack bean, yielded peptide fragments which served as substrates for glycosylation. These results suggests that for some proteins, disruption of the tertiary structure is sufficient to allow attachment of carbohydrate. Other denatured proteins may possess additional restrictions imposed by their secondary structure. In certain cases, these restrictions are removed when the polypeptide chain is fragmented.

Key words: glycosylation, lipid-linked saccharides, glycoproteins, oligosaccharides

It is now well established that saccharides linked to dolichol pyrophosphate participate as intermediates in the synthesis of the carbohydrate chains of certain N-glycosidically linked membrane (1–3) and secretory proteins (4–7). Relatively little is known about the structural requirements of the protein acceptor, although it has been clear for a number of years that in glycoproteins containing carbohydrate N-glycosidically linked to an ASN residue the sequence following the ASN invariably is -X-SER- or -X-THR- (8).

Recently an approach to gaining further insight into the structural requirement of protein acceptors became available when it was found that 2 proteins of known sequence (RNase A and α-lactalbumin) containing the tripeptide sequences -ASN-X-(SER/THR)-

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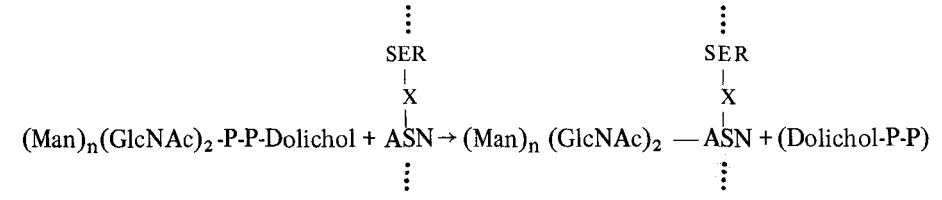
were shown to serve as acceptors of the oligosaccharide chain of oligosaccharide-P-P-dolichol (9). The reaction is believed to proceed as shown below:



Of particular interest was the finding that both RNase and α-lactalbumin served as acceptors only after covalent modifications of all cysteine residues under denaturing conditions (9). However, this treatment did not convert all the proteins tested that contained the sequence -ASN-X-(SER/THR)- into active acceptors.

In the present study we have begun to characterize the requirements of the hen oviduct enzyme(s) involved in the transfer of carbohydrate from GDP-Man to exogenous acceptor proteins via oligosaccharide-lipid, and have attempted to gain information about the structural features of proteins that may regulate their ability to serve as carbohydrate acceptors. A variety of proteins of known amino acid sequence which contain 1 or more of the appropriate asparagine glycosylation sites (10–12), but which do not normally exist in a glycosylated form, have been tested as substrates. Of 7 proteins tested, 2 (rabbit muscle triosephosphate isomerase and ovine prolactin) serve to accept oligosaccharide chains from oligosaccharide-lipid after the tertiary structure of the protein has been disrupted by reduction and alkylation. Of 5 proteins that do not accept carbohydrate after such denaturation, 2 (catalase and concanavalin A) have been cleaved by treatment with cyanogen bromide. Experiments with the resulting mixture of polypeptide fragments indicate that, unlike the intact polypeptide, these fragments do serve as oligosaccharide acceptors.

MATERIALS AND METHODS

Catalase (2X crystallized, bovine liver); elastase (Type III, porcine pancreas); glyceraldehyde-3-phosphate dehydrogenase (Baker's yeast); α-lactalbumin (Grade II, bovine milk); prolactin (ovine pituitary); triosephosphate isomerase (Type III, rabbit muscle); trypsinogen (Type I, bovine pancreas); and iodoacetic acid (sodium salt) were all products of the Sigma Chemical Corporation, St. Louis, Missouri. Concanavalin A (2X crystallized, jack bean) was purchased from Miles Laboratories, Inc, Kankakee, Illinois. Ethylene imine was obtained from the Pierce Chemical Company. Ovine prolactin (lot number NIH-P-512), prepared and characterized by the National Institute of Arthritis, Metabolism and Digestive Diseases, was kindly provided by Mr. T. Anderson and Dr. K. E. Ebner, University of Kansas Medical Center, Kansas City, Kansas. Tunicamycin was the gift of Dr. G. Tamura, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

GDP[^{14}C] Mannose (210 mCi/mmole) was obtained from the New England Nuclear Corporation, Boston, Massachusetts and UDP-GlcNAc was purchased from Boehringer-Mannheim Corp. White leghorn laying hens (26–38 weeks old) were purchased from Truslow Farms, Inc., Chestertown, Maryland.

Before derivitization, proteins were dialyzed against distilled water for 12 h to remove salts and stabilizing agents and lyophilized. The residue was dissolved in 6 M guanidine-HCl or in 8 M urea, reduced, and subjected to either S-carboxymethylation (13), or to S-aminoethylation (14), followed by dialysis against distilled water for 3–4 days. The derivatized proteins dissolved readily in water at concentrations of 7–10 mg/ml when the pH was adjusted to 7.2–7.5 by the addition of NaOH. Cyanogen bromide cleavage was performed on lyophilized samples of the carboxymethylated proteins after they were dissolved in 70% formic acid (vol/vol) (14). After incubation for 24 h at room temperature excess reagents were removed by passing the sample over a Biogel P-4 collumn (1 × 19 cm) equilibrated with 50% formic acid (vol/vol). The peptides eluting from the column in the void volume fractions were pooled. The pooled material was diluted to a final concentration of 1% formic acid and lyophilized to dryness.

The oviduct enzyme preparation used in these experiments was a crude membrane fraction isolated by modification of a previously published procedure (5). It was stored at −20°C prior to use. Protein was measured by the method of Lowry et al. (15).

The enzymatic conversion of exogenous native or derivitized proteins to [^{14}C]-mannose-labeled glycoproteins was assayed as follows: Standard reaction mixtures contained 600–800 μg of oviduct membrane protein, 100–400 μg of exogenous protein, 20 mM Tris-HCl, pH 7.5, 64 mM sucrose, 64 mM NaCl, 4 mM $MgCl_2$, 250 μM UDP-N-acetylglucosamine and either 8 μM GDP[^{14}C]mannose (3×10^6 cpm) or [Man-^{14}C]-oligosaccharide-lipid (2×10^4 cpm) prepared as previously described (16) in a total volume of 120 μl. After incubation at 37°C for 1 h the reaction mixtures were centrifuged at 27,000 × g for 10 min and aliquots of the supernatants and pellets were analyzed by disk gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), urea, and β-mercaptoethanol on 7.5% or 15% polyacryalmide gels (16). After electrophoresis, the gels were stained for protein with Coomassie blue and were then cut into 1.2-mm slices. Gel slices were extracted in capped scintillation vials for 12 h at 65°C with 0.7 ml of 0.1 N NaOH and 1% SDS. Samples were neutralized with HCl and radioactivity was measured after addition of 10 ml of Hydromix (Yorktown Research) using a Packard Tri-Carb liquid scintillation counter. Under these conditions 85–90% of the trichloroacetic acid (TCA) precipitable radioactivity applied to gels was recovered.

A more rapid procedure was also used to assess the conversion of exogenously added protein to [^{14}C]mannose-labeled glycoprotein. Analysis by SDS-gel electrophoresis indicated that approximately 90% of the glycosylated exogenous protein was present in the supernatant after the reaction mixture was centrifuged at 27,000 × g, while most of the radioactive endogenous membrane proteins remained in the pellet. Supernatants from centrifuged reaction mixtures were added to test tubes containing 3 ml of 10% TCA and 1 mg of bovine serum albumin as carrier protein. The tubes were boiled for 10 min to hydrolyze and render acid-soluble GDP[^{14}C]Man and any [Man-^{14}C]oligosaccharide-lipid present in the assay supernatants. The tubes were chilled on ice, centrifuged, and TCA-insoluble pellets were washed 2 additional times with 5-ml aliquots of cold 10% TCA. The pellets were dissolved in 10 ml of Hydromix and radioactivity was measured. An increase in TCA-precipitable radioactivity in supernatants from assays containing exogenous protein, compared to supernatants from control assays containing only oviduct membrane protein, indicated that exogenously added protein was being enzymatically glycosylated, though it was always necessary to confirm the identity of the radioactive protein product on polyacrylamide gels.

RESULTS

Survey of Ability of Derivitized Proteins to Accept Carbohydrate

A simple TCA precipitation assay (described in Materials and Methods) was initially used to determine the ability of a series of derivitized proteins to serve as oligosaccharide acceptors based on the following observation. When an assay containing denatured α-lactalbumin, a known carbohydrate acceptor (9), was centrifuged at 27,000 × g for 10 min and the supernatant and pellet were analyzed separately by electrophoresis on SDS-polyacrylamide gels, approximately 90% of the total [^{14}C] mannose-labeled α-lactalbumin was recovered in the supernatant, while the majority of the labeled endogenous membrane proteins remained in the pellet. The degree to which the addition of other exogenous proteins resulted in enhanced incorporation of [^{14}C] mannose into the TCA-precipitable fraction in assay supernatants (relative to the background of endogenous incorporation) was used as a preliminary indication of their ability to serve as oligosaccharide acceptors.

A list of the proteins tested, their monomeric molecular weights, and the number of -ASN-X-(SER/THR)- tripeptides each contains, is presented in Table I. The subscript number by each amino acid refers to its position in the published sequence (10–12). Derivitized proteins (200 μg) were incubated in the standard incubation mixture containing GDP[^{14}C] mannose. Assays were centrifuged at 27,000 × g for 10 min and TCA-precipitable radioactivity in supernatants was measured and compared to control assays containing no exogenous protein. A parallel assay containing a known acceptor protein, S-carboxymethylated α-lactalbumin, was done to monitor the activity of the enzyme preparation. The results of such a survey (Table II) showed that, like α-lactalbumin, the addition of 2 other S-carboxymethylated proteins, triosephosphate isomerase and prolactin, to reaction mixtures resulted in a considerable increase in incorporation of radioactivity into TCA-precipitable protein in the supernatant fraction. On the basis of this

TABLE I. Potential Carbohydrate Acceptor Proteins

Protein	Molecular weight (monomers)	Potential site of carbohydrate attachment
Catalase (10)	61,000	-ASX_{212}-THR_{213}-SER_{214}-[a] -ASN_{242}-LEU_{243}-SER_{244}-[a] -ASN_{437}-VAL_{438}-THR_{439}-[a] -ASN_{479}-PHE_{480}-SER_{481}-[a]
Concanavalin A (12)	25,572	-ASN_{118}-SER_{119}-THR_{120}- -ASN_{162}-GLY_{163}-SER_{164}-
Elastase (10)	25,906	-ASN_{66}-GLY_{67}-THR_{68}- -ASN_{123}-ASN_{124}-SER_{125} -ASN_{215}-VAL_{216}-THR_{217}-
Glyceraldehyde-3-phosphate-dehydrogenase (11)	35,549	-ASN_{146}-ALA_{147}-SER_{148}- -ASX_{236}-VAL_{237}-SER_{238}-
α-Lactalbumin (10	14,183	-ASN_{45}-GLN_{46}-SER_{47}- -ASN_{74}-ILE_{75}-SER_{76}-
Prolactin (10)	22,554	-ASN_{31}-LEU_{32}-SER_{33}-
Triosephosphate isomerase (12)	26,626	-ASN_{195}-VAL_{196}-SER_{197}-
Trypsinogen (10)	23,990	-ASN_{151}-SER_{152}-SER_{153}-

[a]In the case of catalase, for which only fragments have been sequenced, numbers refer to the position of amino acids in the fragment (10), not to the absolute position of these residues in the polypeptide chain.

TABLE II. Incorporation of Mannose From GDP[^{14}C]Mannose Into Exogenous Derivitized Proteins*

	Radioactivity incorporated (cpm)	Incorporation relative to control (no exogenous protein)
Carboxymethylated proteins		
None	9,170	(1.0)
α-Lactalbumin	35,450	3.9
Triosephosphate isomerase	36,500	4.0
Prolactin	17,530	1.9
Catalase	13,500	1.5
Elastase	9,030	1.0
Glyceraldehyde-3-phosphate dehydrogenase	8,230	0.9
Concanavalin A	4,400	0.5
Trypsinogen	5,820	0.6
Aminoethylated proteins		
None	5,860	(1.0)
α-Lactalbumin	26,480	4.5
Prolactin	11,322	1.9
Catalase	2,760	0.5
Elastase	3,420	0.6
Glyceraldehyde-3-phosphate dehydrogenase	6,950	1.2
Concanavalin A	3,110	0.5
Trypsinogen	3,590	0.6

*S-carboxymethylated and S-aminoethylated derivatives of proteins were prepared as described in Materials and Methods. Each derivitized protein (200 μg) was tested for carbohydrate acceptor ability by incubation under standard incubation conditions. After incubation for 60 min glycosylated protein in the supernatant was measured as described in Materials and Methods.

experiment S-carboxymethylated catalase was considered a questionable carbohydrate acceptor since it afforded only a slight increase in radioactivity. In the presence of S-carboxymethylated elastase or glyceraldehyde-3-phosphate dehydrogenase radioactivity was incorporated in supernatants at a level no greater than that of endogenous incorporation, whereas the presence of S-carboxymethylated concanavalin A or trypsinogen suppressed incorporation of [^{14}C]mannose to approximately half that of the endogenous level. These results indicate that the latter 4 proteins were inactive as carbohydrate acceptors under the conditions described.

The S-aminoethylated derivatives of the same proteins were assayed to determine if the introduction of a positively charged aminoethyl group in place of a negatively charged carboxymethyl group would have an effect on the carbohydrate acceptor capacity. The results, also presented in Table II, indicate that the addition of α-lactalbumin or prolactin again resulted in an increase of radioactivity into the supernatant fraction. S-Aminoethylated triosephosphate isomerase was not tested. The aminoethyl derivatives of catalase as well as the aminoethyl derivatives of the other 4 proteins found to be inactive as carboxymethyl derivatives were all inactive.

To confirm the results shown in Table II, the radioactive products formed with the derivitized proteins were analyzed by electrophoresis on SDS-polyacrylamide gels. The supernatant fractions from assays containing S-carboxymethylated α-lactalbumin, prolactin, or triosephosphate isomerase all contained a new radioactive polypeptide which had an electrophoretic mobility slightly slower than that of the respective unlabeled

derivatized protein (Fig. 1 A, C, and E). As previously observed (9), this slower mobility is consistent with the increased molecular weight expected after labeled oligosaccharide is incorporated into protein. When equivalent amounts of native α-lactalbumin, prolactin, or triosephosphate isomerase were assayed, the new radioactive peaks were absent or greatly reduced (Fig. 1 B, D, and F), indicating that these proteins are essentially incapable of serving as carbohydrate acceptors unless their tertiary structure has been disrupted.

SDS-polyacrylamide gel electrophoresis indicated that no new radioactive polypeptides were present in either the supernatants or the pellets from assays containing the S-carboxymethylated forms of catalase, concanavalin A, elastase, glyceraldehyde-3-phosphate dehydrogenase, trypsinogen, or S-aminoethylated glyceraldehyde-3-phosphate dehydrogenase (data not shown). It was concluded that, even though these 5 proteins

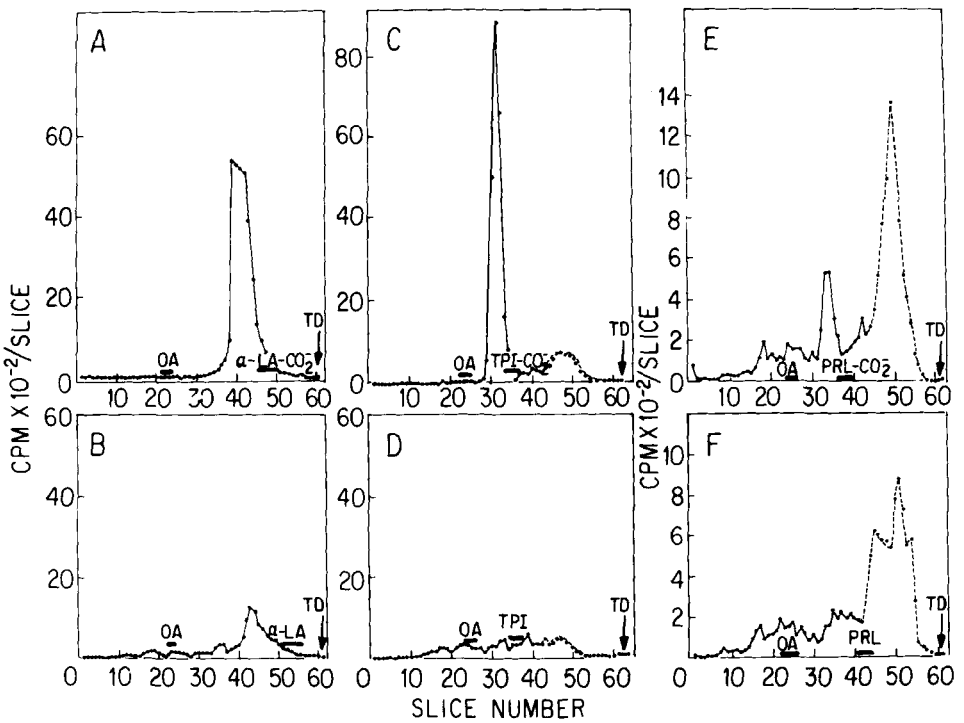


Fig. 1. Electrophoretic analysis of [^{14}C] mannose-labeled polypeptides synthesized from exogenously added native or S-carboxymethylated proteins. Native and derivitized proteins were tested for their ability to accept carbohydrate using standard assay conditions. Proteins added were: A) S-carboxymethylated α-lactalbumin (α-LA-CO_2^-) or B) native α-lactalbumin (α-LA), 200 μg (14 nmoles); C) S-carbosymethylated triosephosphate isomerase (TPI-CO_2^-) or D) native triosephosphate isomerase (TPI), 200 μg (7.5 nmoles); E) S-carboxymethylated prolactin (PRL-CO_2^-) or F) native prolactin (PRL), 200 and 400 μg respectively (9 nmoles and 17 nmoles). After incubation at 37°C for 60 min the reactions were centrifuged at 27,000 × g for 10 min. Aliquots of the supernatant (60 μl) were analyzed by electrophoresis on 7.5% polyacrylamide disk gels in the presence of 1% SDS, 0.5 M urea, and 2% β-mercaptoethanol. The positions of ovalbumin (OA, present endogenously in the oviduct enzyme preparation), native exogenous proteins (α-LA, TPI, PRL) and S-carboxymethylated exogenous proteins (α-LA-CO_2^-, TPI – CO_2^-, PRL – CO_2^-) are shown by the heavy bars. Peaks denoted by a dashed line represent the variable amount of [^{14}C] Man-P-dolichol and [Man-^{14}C] oligosaccharide-lipid recovered in the supernatant. TD marks the front of the tracking dye.

contain at least 1 of the appropriate asparagine acceptor sites, neither the S-carboxymethylated nor the S-aminoethylated derivatives could be glycosylated under the stated assay conditions.

Because of the expense of preparing and utilizing isolated [Man-^{14}C] oligosaccharide-lipid as the radioactive substrate, the above experiments were performed with [Man-^{14}C] oligosaccharide-lipid generated endogenously by addition of GDP[^{14}C] Man and UDP-GlcNAc. Previous studies (17) have shown that the carbohydrate chain transferred to exogenous S-carboxymethylated α-lactalbumin is identical, whether the substrate is preformed, exogenous [Man-^{14}C] oligosaccharide-lipid, or endogenously generated oligosaccharide-lipid. To determine that S-carboxymethylated prolactin and triosephosphate isomerase were indeed glycosylated via saccharide-lipid intermediates, the effect of tunicamycin on glycosylation of these 2 proteins was studied. Work by others (18–21) has shown that tunicamycin specifically inhibits synthesis of the GlcNAc-P-P-dolichol lipid that serves as the acceptor for synthesis of $(Man)_X$ GlcNAcGlcNAc-P-P-dolichol (22). Moreover, using whole cells or slices, it has been shown that tunicamycin blocks glycosylation of a variety of secretory proteins (5–7). As shown in Table III the transfer of mannose from GDP-[^{14}C] mannose to endogenous membrane protein or exogenous derivitized protein was virtually abolished in the presence of the antibiotic tunicamycin. To further corraborate this finding, which indicates that oligosaccharide-lipid does participate in the glycosylation, incubations were carried out in which S-carboxymethylated prolactin or triosephosphate isomerase were incubated with [Man-^{14}C] oligosaccharide-lipid instead of GDP[^{14}C] mannose and UDP-GlcNAc. The incubation supernatants were found to contain labeled proteins having the same mobility as those synthesized using GDP[^{14}C] mannose (data not shown), though the yields were much lower due to the limited amounts of [Man-^{14}C] oligosaccharide-lipid substrate available.

Effect of Membrane Enzyme Concentration, Substrate Concentration and pH on Transfer of Mannose From GDP[^{14}C] Mannose to S-Carboxymethylated Proteins.

Since, with the exception of our early study utilizing sulfitolyzed RNase A as acceptor (9), little was known about the effect of incubation conditions on the extent of glycosylation of exogenous protein acceptors, several variables were examined. As shown

TABLE III. Effect of Tunicamycin on Incorporation of Mannose From GDP[^{14}C] Mannose Into Endogenous and Exogenous Protein*

Addition	Radioactivity incorporated (cpm)		Inhibition of [^{14}C] mannose incorporation (%)
	Minus tunicamycin	Plus tunicamycin	
None	12,730	1,050	92
Carboxymethylated α-lactalbumin	45,500	2,070	96
Carboxymethylated prolactin	22,660	1,330	94
Carboxymethylated triosephosphate isomerase	36,130	1,410	96

*Standard reaction mixtures containing 200 μg of the specified S-carboxymethylated proteins were incubated for 60 min in the absence or presence of tunicamycin at a final concentration of 2 μg/ml. The tunicamycin was dissolved in 0.01 N NaOH; samples which were not treated with tunicamycin received an equivalent volume of 0.01 N NaOH. Glycosylated protein in the supernatant was measured as described in Materials and Methods

in Fig. 2A, there was a linear dependence between the amount of membrane enzyme added and the incorporation of [^{14}C] mannose from GDP[^{14}C] mannose into exogenous derivitized α-lactalbumin. Using a fixed amount of membrane enzyme a linear dependence between the amount of exogenous protein added and incorporation of TCA-precipitable radioactivity into supernatants was seen using derivitized α-lactalbumin, or triosephosphate isomerase (Fig. 2B). The transfer of mannose from GDP[^{14}C] mannose to S-carboxymethylated α-lactalbumin was stimulated up to eightfold by the addition of unlabeled UDP-N-acetyl-glucosamine (Fig. 2C), which is consistent with our earlier findings that glycosylation was

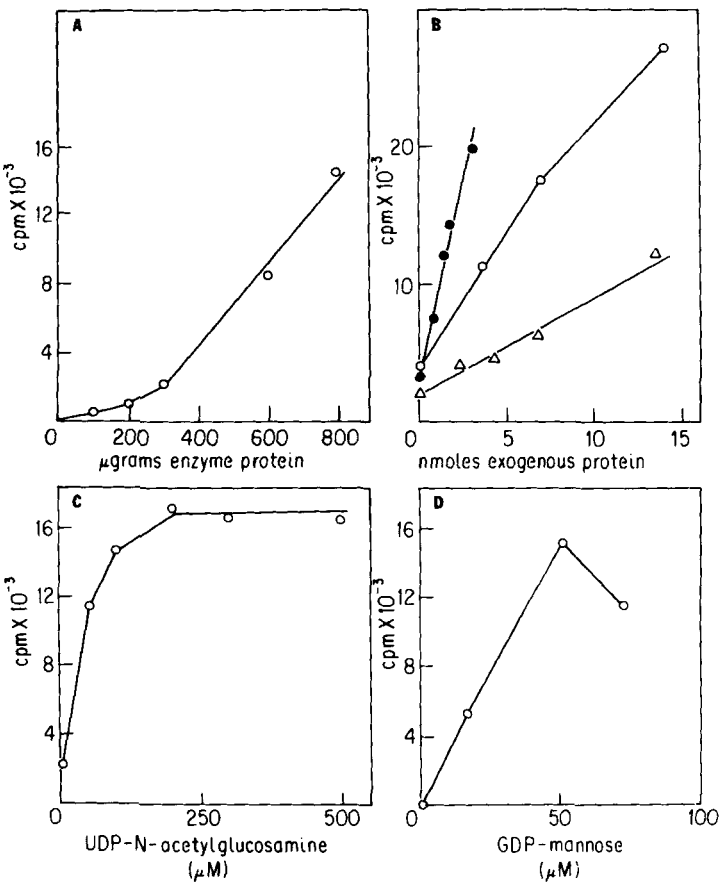


Fig. 2. Effect of enzyme and substrate concentration on glycosylation of S-carboxymethylated proteins. A) Dependence on enzyme protein. Assays contained 20 mM Tris-HCl, pH 7.5, 64 mM sucrose, 64 mM NaCl, 4 mM $MgCl_2$, 180 μg (12 nmoles) of S-carboxymethylated α-lactalbumin, 0-800 μg of oviduct enzyme protein, 250 μM UDP-N-acetylglucosamine, and 8 μM GDP[^{14}C] mannose. B) Dependence on exogenous acceptors: standard assays, described in A, contained 700 μg oviduct membrane enzyme, varying amounts of one of the following S-carboxymethylated proteins: triosephosphate isomerase (-•-•-), α-lactalbumin (-○-○-), prolactin (-△-△-), and 8 μM GDP[^{14}C] mannose. C and D) Sugar nucleotide dependence: standard assays, described in A, contained 700 μg of oviduct enzyme protein, 200 μg (14 nmoles) of S-carboxymethylated α-lactalbumin and varying concentrations of UDP-N-acetyl-glucosamine (0–600 μM) (C), or GDP[^{14}C] mannose (0–73 μM), specific radioactivity 21 mCi/mmole (D). After incubation for 35 min at 37°C, assays were centrifuged at 27,000 × g for 10 min and TCA-precipitable radioactivity in the supernatants was analyzed as described in Materials and Methods.

blocked by tunicamycin. Maximal sugar incorporation was seen at a UDP-N-acetylglucosamine concentration of 200 μM. As expected, increasing amounts of GDP-mannose (up to 50 μM) also had an increase on mannose incorporation into protein (Fig. 2D). Above 50 μM GDP-mannose, an inhibitory effect was seen, possibly due to end product inhibition by GDP.

As shown in Fig. 3, the optimal pH for glycosylation varies, depending on the exogenous protein used as carbohydrate acceptor. The pH optima for glycosylation of α-lactalbumin (pH 6.4) and triosephosphate isomerase (pH 7.0) are clearly different from the optima for glycosylation of endogenous membrane protein (pH 7.5).

It was clear from this study, as well as an earlier one (9), that a number of proteins containing the necessary -ASN-X-(SER/THR)- tripeptide could not be glycosylated, even after reduction and alkylation. One possible explanation was that, although the acceptor asparagine in the inactive proteins might be accessible to the transferase enzyme(s), additional essential "information" residing in some other region of the polypeptide chain

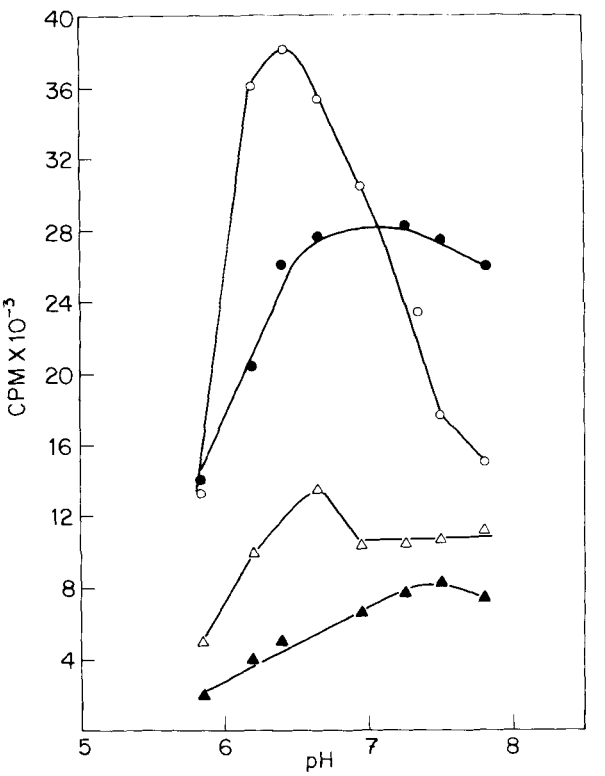


Fig. 3. Effect of pH on glycosylation of S-carboxymethylated proteins. Assays contained 50 mM Tris-maleate buffer at the indicated pH, 64 mM sucrose, 4 mM $MgCl_2$, 250 μM UDP-N-acetylglucosamine, 8 μM GDP[^{14}C] mannose, and either no additions (-▲-▲-) or the following amounts of S-carboxymethylated exogenous protein: α-lactalbumin (-○-○-), 113 μg (8 nmoles); triosephosphate isomerase (-●-●-) 117 μg (4 nmoles); or prolactin (-△-△-), 118 μg (5 nmoles). After incubation for 30 min at 37°C, reaction mixtures were centrifuged at 27,000 X g for 10 min and TCA-precipitable radioactivity in the supernatants was analyzed as described in Materials and Methods.

was lacking. An alternative explanation was that perhaps even though the tertiary structure of all these proteins had been disrupted, in the case of the inactive ones, the appropriate asparagine acceptor residue might still be inaccessible due to restrictions on the protein imposed by remaining domains of ordered structure.

We reasoned that if indeed the latter explanation was valid, cleavage of the protein might yield a smaller peptide which would serve as a substrate for glycosylation. Consequently, cyanogen bromide cleavage fragments from S-carboxymethylated catalase and concanavalin A were prepared and assayed for carbohydrate acceptor ability using the TCA precipitation assay. The results, presented in Table IV, demonstrate that cyanogen bromide cleavage fragments from catalase and concanavalin A significantly stimulated the incorporation of mannose into TCA-precipitable material. In contrast, intact, native or derivitized catalase and concanavalin A did not serve as acceptors. The incorporation of mannose into the cyanogen bromide fragments from catalase and concanavalin A was inhibited 98% by tunicamycin.

The labeled products formed with the mixture of cyanogen bromide cleavage fragments from S-carboxymethylated catalase and concanavalin A, as well as the native and derivitized proteins from which these fragments are prepared were analyzed by electrophoresis on 15% polyacrylamide gels. The results (Fig. 4) show that, relative to the profile of proteins labeled in the absence of exogenous acceptor (Fig. 4D and H), no new mannose-labeled macromolecules were present in assays containing native catalase or concanavalin A (Fig. 4C and G) or the S-carboxymethylated derivatives of these proteins (Fig. 4B and F). However, distinctive new peaks were formed in the presence of cyanogen bromide cleavage fragments from both catalase and concanavalin A (Fig. 4A and E).

TABLE IV. Incorporation of Mannose From GDP[^{14}C] Mannose Into Exogenous Proteins and Polypeptide Fragments*

Addition	Radioactivity incorporated (cpm)	Incorporation relative to control (minus exogenous protein)
Experiment I		
None	5,850	(1.0)
Catalase	5,690	1.0
S-carboxymethylated catalase	7,000	1.2
CNBr fragments from S-carboxymethyl catalase	14,720	2.5
S-carboxymethylated α-lactalbumin	20,000	3.4
Experiment II		
None	3,360	(1.0)
Concanavalin A	1,340	0.4
S-carboxymethyl concanavalin A	1,260	0.4
CNBr fragments from S-carboxymethyl concanavalin A	6,610	2.0
S-carboxymethyl α-lactalbumin	12,870	3.8

*Standard reaction mixtures containing 200 μg of native protein, 200 μg of S-carboxymethylated proteins, or 200 μg of unfractionated cyanogen bromide cleavage fragments prepared from S-carboxymethylated proteins were incubated for 60 min. Glycosylated polypeptides in the supernatant were assayed as described in Materials and Methods.

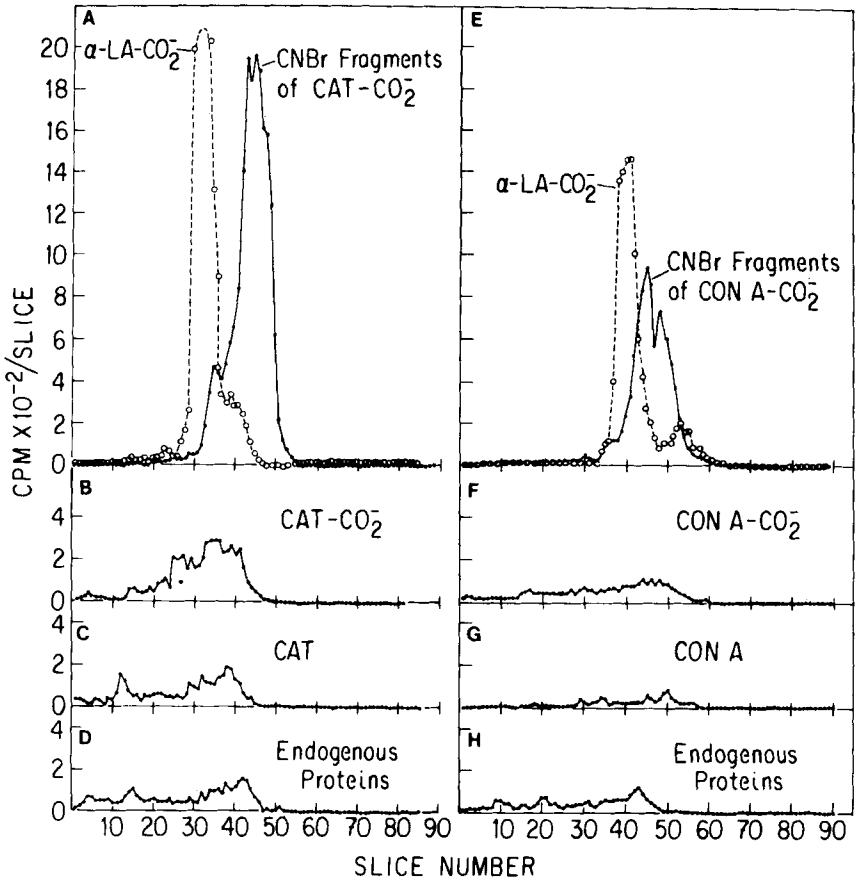


Fig. 4. Electrophoretic analysis of [^{14}C] mannose labeled polypeptides synthesized in the presence of exogenous proteins or polypeptide fragments. The following polypeptides were added to the standard reaction mixture: A) 200 μg of unfractionated cyanogen bromide cleavage fragments from S-carboxymethyl catalase; B) 200 μg of S-carboxymethyl catalase (Cat-CO_2^-); C) 200 μg of native catalase (Cat); D) no exogenous protein added; E) 200 μg of unfractionated cyanogen bromide cleavage fragments from S-carboxymethyl concanavalin A; F) 200 μg of S-carboxymethyl concanavalin A (Con A-CO_2^-); and G) no exogenous protein added. After 60 min at 37°C the reactions were centrifuged at 27,000 × g for 10 min. Aliquots of the supernatants (60 μl) were analyzed on 15% polyacrylamide disk gels in the presence of 1% SDS, 0.5 M urea, and 2% β-mercaptoethanol. The dashed lines in A and E represent the electrophoretic profile of the glycosylated product obtained from an identical assay containing 200 μg of S-carboxymethyl α-lactalbumin (α-LA-CO_2^-), and the dark bars show the position of catalase (Cat) and concanavalin A (Con A). The arrow at the top of the figures marks the front of the tracking dye (TD).

Because of the lack of resolution obtained on 15% gels it was difficult to determine whether these new peaks contained a single or multiple components, but it is clear that they are lower in molecular weight than reference α-lactalbumin (mol. wt. 14,200). According to the published amino acid sequences of these proteins (10–12), tripeptide acceptor sequences are present in 2 cyanogen bromide cleavage fragments from catalase. One fragment (mol. wt. 13,500) has 2 potential acceptor sites; the other fragment (mol. wt. 79,400) contains 1 site or possibly 2, depending on whether amino acid residue 212 is

asparagine or aspartate. The potential acceptor sites in concanavalin A are also present in 2 separate cyanogen bromide cleavage fragments of mol. wt. 10,600 and 13,300.

To obtain better resolution of the new glycosylated products formed in the presence of the cyanogen bromide cleavage fragments, gel filtration on Sephadex G-100 was used. The results (Fig. 5) clearly show that at least 2 distinct products are formed in assays containing the cyanogen bromide cleavage fragments from both catalase (Fig. 5A) or concanavalin A (Fig. 5B). No such products were found in the presence of endogenous oviduct proteins (Fig. 5C). The included volume from these columns contained unreacted GDP-[^{14}C] mannose and its breakdown products; the small, partially included peak is free [Man-^{14}C] oligosaccharide. Peaks I and II from both the catalase and concanavalin A fragments were pooled separately and the TCA-precipitable radioactivity in these fractions was determined before and after extensive pronase digestion (24). Pronase rendered over 95% of the radioactivity in the 4 fractions TCA-soluble, indicating that these new products are indeed glycopeptides.

DISCUSSION

Using a simple TCA precipitation assay and a membrane fraction from hen oviduct we have surveyed a variety of denatured proteins and peptides having 1 or more tripeptide sequences of the structure -ASN-X-(SER/THR)- for their ability to accept the oligosaccharide chain of [Man-^{14}C] oligosaccharide- P-P-dolichol. Based on the earlier finding (17) that identical mannose-labeled glycoproteins are synthesized whether GDP[^{14}C] mannose or [Man^{14}C] oligosaccharide-lipid are used as the sugar donor, the assay for glycosylation of exogenous proteins has been simplified and made more economical. In the presence of GDP[^{14}C] mannose and UDP-N-acetylglucosamine, endogenous enzymes in the oviduct membrane preparation catalyze both the synthesis of [Man-^{14}C] oligosaccharide-lipid and the transfer of its oligosaccharide chain to certain exogenous proteins which are readily recovered from the reaction mixture. Inhibition by tunicamycin of mannose incorporation indicates that under the assay conditions used virtually all mannose transfer (95%) into protein is dependent on the synthesis of N-acetylglucosamine-lipid intermediates.

In addition to the previously reported derivatives of α-lactalbumin, ribonuclease A and ovalbumin (9), 2 other exogenous proteins, triosephosphate isomerase and prolactin have been shown to function as oligosaccharide acceptors after reduction and derivitization. In addition, in experiments not shown S-carboxymethylated and S-aminoethylated DNase were found to serve as acceptors under the assay methods used in this study (D. Struck and W.J. Lennarz, unpublished studies). In an earlier study (9) the sulfitolyzed derivatives of elastase, carboxypeptidase, DNase, and alcohol dehydrogenase, all of which contain one or more -ASN-X-(SER/THR)- sequences, were shown to be inactive as carbohydrate acceptors. Based on the current study this list now can be enlarged to include the S-carboxymethyl and S-aminoethyl derivatives of catalase, concanavalin A, elastase, glyceraldehyde-3-phosphate dehydrogenase, and trypsinogen. Thus, of 13 potential carbohydrate acceptor proteins tested so far, 6 have been found to serve as acceptors.

One possible explanation for the inactivity of the remaining 7 proteins is that the charge introduced by derivitization prevents interaction of the protein with membrane-bound oligosaccharide transferase. However, this seems unlikely for several reasons. First, neither the carboxymethyl derivatives (negative charge) nor the aminoethyl derivatives (positive charge) of these 7 proteins served as acceptors. Second, although the carboxy-

methyl and aminoethyl derivatives of the active proteins quantitatively differed in their ability to accept the oligosaccharide, in all cases both types of derivatives could be shown to be glycosylated.

As shown in the pH dependence studies, pH, and therefore charge, does play a role in the rate of glycosylation. Moreover, the pH affects not only the membrane-bound enzyme system but also the acceptor protein, because the optimal pH for glycosylation

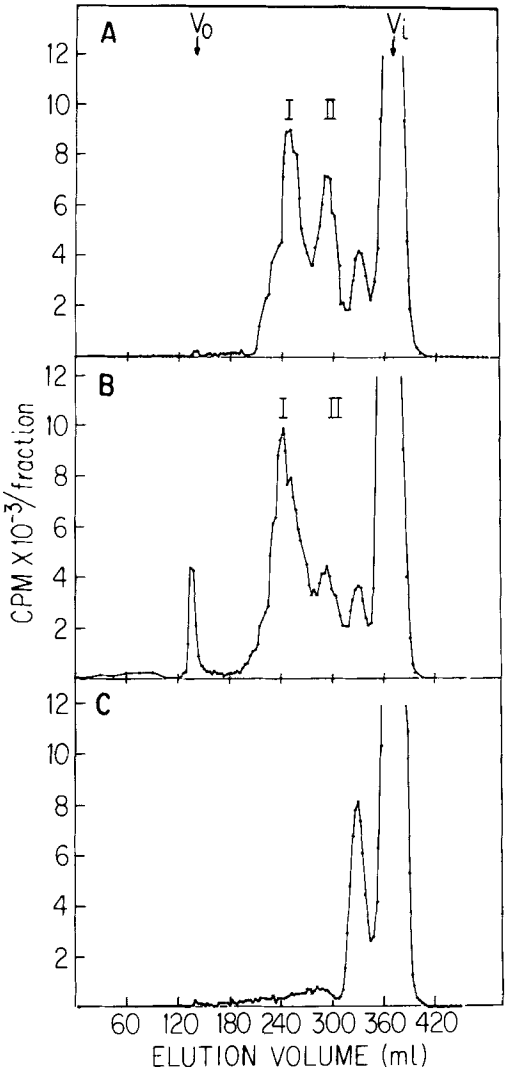


Fig. 5. Analysis of glycosylated polypeptide fragments by gel filtration on Sephadex G-100. Reaction products from standard assays as described in Fig. 4 were applied to a Sephadex G-100 column and eluted with 0.1 M NH_4HCO_3. A) Cyanogen bromide cleavage fragments from S-carboxymethylated catalase; B) cyanogen bromide cleavage fragments from S-carboxymethylated concanavalin A; or C) no exogenous protein. Arrows mark the elution position of the void volume (V_0) and the included volume (V_i).

depends on the particular acceptor protein tested. Nevertheless, under no conditions could the inactive proteins listed above be glycosylated.

Based on these findings, we have considered 2 possible explanations for the finding that certain proteins containing -ASN-X-(SER/THR)- serve as substrates, while others with this tripeptide do not. One possibility is that specific amino acid residues, either in position "X" or in the sequences adjacent to the tripeptide, are required for a protein to be active. Examination of the sequences of both the active and inactive proteins does not reveal any sequence features consistent with this interpretation. The other possibility is that the inactive, denatured proteins still retain regions of order that, in some way, make the acceptor tripeptide sequence unavailable for interaction with the transferase.

If the latter explanation is correct, one would expect that if the size of the polypeptide chain of the inactive proteins was reduced by chemical or enzymatic cleavage, the previously inaccessible tripeptide might become exposed. The results of the current study, showing that a mixture of fragments of catalase, and of concanavalin A, generated by CNBr cleavage are able to accept the oligosaccharide, are consistent with this idea. Moreover, more extensive studies with isolated, purified polypeptide fragments of α-lactalbumin and RNase (D. Struck, G. Hart, K. Brew, G. Grant, R. Bradshaw, and W. J. Lennarz, unpublished studies) show that large portions of the polypeptide chain of active acceptors can be removed without any loss of acceptor ability. Thus, at present it appears the presence of the tripeptide -ASN-X-(SER/THR)- may be sufficient for a protein to be glycosylated, provided that this site is accessible for interaction with the enzyme. We are currently testing this hypothesis by preparing small peptides containing the sequence -ASN-X-SER- or --ASN-X-THR-.

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