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^{13}C-N.M.R.-SPECTRAL STUDY OF GALACTOSYLTRANSFERASE SPECIFICITY WITH REGARD TO THE CARBOHYDRATE SIDE-CHAIN OF NATIVE OVALBUMIN*

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ABSTRACT

As a prelude to studies using bovine *N*-acetylglucosaminide-β-(1→4)-galactosyltransferase to label membrane-surface glycoproteins with isotopically enriched D-galactose, the structural specificity of the enzymic reaction with water-soluble, hen ovalbumin has been examined. The enzyme-catalyzed transfer of D-galactose from UDP-D-galactose requires a (nonreducing) terminal 2-acetamido-2-deoxy-D-glucosyl group and exhibits selectivity towards saccharide chains containing D-mannose. This study considers the structural specificity of the enzyme with regard to the anomeric linkage between 2-acetamido-2-deoxy-D-glucose and D-mannose in the carbohydrate chains of hen ovalbumin. Uniformly ^{13}C-enriched D-galactose was enzymically attached to the ovalbumin carbohydrate chain (which exhibits microheterogeneity in its structure), the protein was hydrolyzed, and separate glycopeptide fractions were chromatographically isolated. The ^{13}C-n.m.r. spectra (60.5 MHz) of the fractions revealed two peaks for the anomeric carbon atom of D-galactose. The two peaks, at 104.20 and 104.39 p.p.m., were ascribed to D-galactosyl groups attached to 2-acetamido-2-deoxy-D-glucose respectively linked β-(1→4) and β-(1→2), to D-mannose in the glycopeptide chains. Quantifying of the spectral data revealed no specificity of D-galactosyltransferase towards the linkage from the terminal 2-acetamido-2-deoxy-D-glucosyl group to the penultimate D-mannosyl residue.

INTRODUCTION

The galactosyltransferases are among the most studied groups of mammalian glycosyltransferases, mainly because galactosyltransferase activity has been found in essentially every cell and tissue, as well as in a variety of extracellular fluids[1,2].

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N-Acetylglucosaminide-β-(1→4)-galactosyltransferase is a particularly well studied enzyme as homogeneous preparations of it are relatively easy to obtain[1]. The transfer reaction requires a nonreducing, terminal β-D-GlcNAc group.

$$\text{UDP-D-Gal} + \text{D-GlcNAc} \xrightarrow[\text{enzyme}]{Mn^{2+}} \beta\text{-D-Gal-(1}\rightarrow\text{4)-D-GlcNAc} + \text{UDP}$$

The transfer is known to occur with a variety of substrates, including glycoproteins and glycopeptides[1,2]. The reaction mechanism and specificity have been studied[1-3]. Multiple binding sites on the enzyme[1], and some sugar specificity, apparently for D-mannose[3], were implicated in these studies. However, there has been no detailed study of the enzyme specificity toward the anomeric linkage between the requisite GlcNAc group and Man residue, *i.e.*, β-D-GlcNAc-(1→2)-α-D-Man *vis-a-vis* β-D-GlcNAc-(1→4)-α-D-Man. The present study was designed to investigate this point with the aid of high-resolution, high-field, ^{13}C-n.m.r. spectroscopy. We have found that the enzyme transfers D-Gal groups both to β-D-GlcNAc-(1→2)-α-D-Man and β-D-GlcNAc-(1→4)-α-D-Man branches of native, hen ovalbumin carbohydrate side-chains, regardless of the overall structure of the side chain.

Another aspect of the study is that the substrate used was a glycoprotein having a complex carbohydrate side-chain, rather than a simple carbohydrate such as is utilized in most enzymic studies. The more-complicated substrate was employed as a more appropriate model for cell-surface glycoproteins, in order to ascertain if the enzyme can be expected to catalyze binding of (labeled) D-Gal to both β-D-GlcNAc-(1→2)- and β-D-GlcNAc-(1→4)-terminated side-chains on cell surfaces. Previously[4], we demonstrated that a ^{13}C-labeled Gal group could be attached (to the carbohydrate chain of hen ovalbumin) by using ^{13}C-labeled D-glucose in a sequence of five enzymic steps, the final step being catalyzed by D-galactosyltransferase (see reaction already shown). Investigation of the specificity of the D-Gal-transfer reaction toward different β-D-GlcNAc linkages is possible, as native hen ovalbumin possesses a large degree of microheterogeneity in the structure of its single carbohydrate side-chain, including terminal β-D-GlcNAc groups linked both (1→2) and (1→4) to Man[5-9]. Because only about half of the possible sites of attachment were utilized by the D-Gal transfer (*vida infra*), there are three possible cases. One is that the D-Gal group was specifically added to either β-D-GlcNAc-(1→2)- or β-D-GlcNAc-(1→4)-; the second is completely random transfer to all linkages; and the third is that of attachment to both anomeric forms, with a preference for one. Using ^{13}C-n.m.r. spectroscopy, we set out to investigate which of these processes occurs and, therefore, to obtain information regarding the thermodynamic specificity of D-galactosyltransferase. The study was accomplished by proteolytic digestion of D-[U-^{13}C]Gal-ovalbumin[4], and examination of the ^{13}C-n.m.r. spectra of the glycopeptide fractions thus formed

EXPERIMENTAL

Materials. — [U-^{13}C]Gal-ovalbumin was the same sample used in ref. 4. Bio-Gel P4-400 was obtained from Bio-Rad Lab., Richmond, CA.; Sephadex G25-300, from Sigma Chemical Co., St. Louis, Mo.; and protease *ex Strep. griseus*, from Cal-Biochem-Behring Corp., La Jolla, CA.

Methods. — The sample (1.1 g) of hen ovalbumin was denatured in 8M urea, dialyzed extensively against double-distilled water to remove the urea, and the solution lyophilized. The dried sample was dissolved in 25 mL of 15mM $CaCl_2$ buffer, pH 8.5, and protease (10 mg) was added. The mixture was constantly stirred, and kept at pH 8.5 and 37°. Three more additions of protease (10 mg each) were made at intervals of ~8 h. The reaction was quenched after 30 h, and the precipitate was removed by centrifugation, and washed twice with water. The supernatant liquors were combined, concentrated *in vacuo* to ~3 mL, and the concentrate placed on a column (2.5 × 55 cm) of Sephadex G-25-300, and eluted with 0.1M acetic acid at a flow rate of ~45 mL/h. The carbohydrate-containing fraction, monitored by the orcinol–sulfuric acid method at 540 nm, was collected, and lyophilized. The recovery of sample, following the protease reaction and gel-filtration chromatography, was >90%.

A solution of the resulting carbohydrate fraction in water was placed on a column (2 × 175 cm) of Bio-Gel P4-400, and eluted with water at a flow rate of 8.5

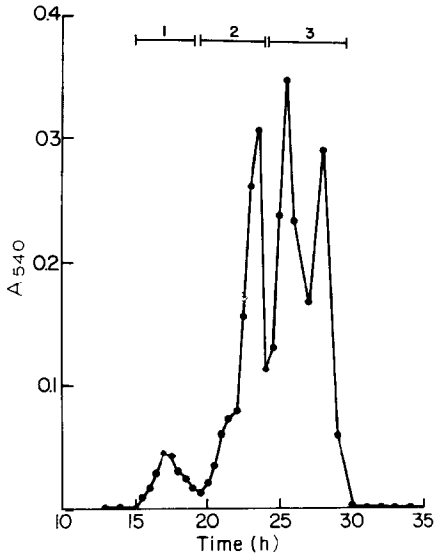


Fig. 1. Gel-filtration chromatography on Bio-Gel P4 of [U-^{13}C]Gal-ovalbumin digest. [The experimental conditions are given in the Experimental section. Fraction 1 probably consists of products of incomplete digestion; fraction 2 corresponds to AC-A, AC-B (the shoulder), and AC-C of ref. 5; fraction 3 contains mainly the "high-D-mannose" carbohydrate structure (AC-D and AC-E in ref. 5) and some minor, "hybrid" carbohydrate structures as found elsewhere[8,10,14].]

mL/h, the effluent being monitored at both 206 and 540 nm. The results of this fractionation are shown in Fig. 1.

The glycopeptides of ovalbumin (without D-Gal-labeling) were previously fractionated[5] by using an ion-exchange column. However, the conditions under which that ion-exchange column was used, namely, at constant ionic strength and pH, resulted in a separation of the glycopeptide fractions on the basis of molecular weight, just as with a gel-permeation column[5-8]. Consequently, the elution profile obtained on fractionating the Gal-labeled ovalbumin (see Fig. 1) should correspond approximately to that obtained earlier[5]. Some deviations would be anticipated, as those fractions having Gal attached should be shifted slightly toward the heavier end of the elution profile. Fractions were pooled into three samples, as indicated by the bars in Fig. 1. The hexose content of each fraction was measured against standardized D-glucose solution, with 1.5, 8, and 15 mg of hexoses being respectively found for fractions 1 to 3. Fractions 2 and 3 were used without purification in the n.m.r. studies.

The ^{13}C-n.m.r. spectra were recorded at 60.5 MHz with a home-built n.m.r instrument using 10-mm n.m.r. tubes. The spectra were acquired in the Ft mode, with 85° pulses, a spectral window of ~12 kHz, 16k memory-data points, and a repetition time of 0.7 s. Line broadening of 2 Hz and zero-fill to 32k memory-data points were used in the processing of the f.i.d.s. Each spectrum is the result of 100,000 accumulations. Lyophilized fractions 2 and 3 were dissolved in identical volumes of D_2O containing the same amount of 1,4-dioxane, which was used as the internal standard both for chemical shifts (67.86 p.p.m. from tetramethylsilane) and intensity. The pH of the samples was in the same range (3.0–3.5), and the temperature was kept at 32°.

RESULTS AND DISCUSSION

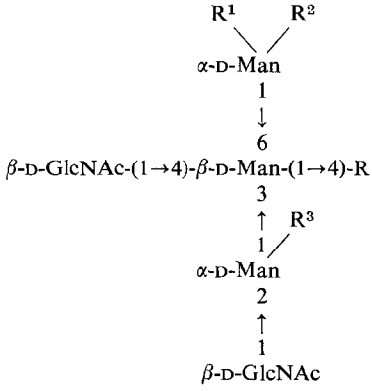
Pooled and single preparations of hen ovalbumin samples are known to exhibit a high degree of structural microheterogeneity of the carbohydrate side-chain, although there is a single point of attachment for this carbohydrate side-chain to the protein. It has been discovered that there are eight different, side-chain structures having at least one (terminal) nonreducing β-D-GlcNAc group[5-10]. These structures are depicted in Table I, which also includes information about their relative proportions in ovalbumin[10]. From these structures, it may be seen that the possible transfer of the D-Gal residue by galactosyltransferase may be affected by the presence of three different types* of terminal β-D-GlcNAc linkage to a penultimate D-mannosyl residue, namely, (1→4), (1→2), and (1→6), and by the overall structure and conformation of each of the different side-chains.

As reported previously[3], the overall carbohydrate structure is implicated in the observed specificity of the enzyme toward ovalbumin. Thus, it seemed conceivable that the enzyme might have structural specificity related to the type of β-D-GlcNAc

*The suggested presence of a (1→6) linkage is tentative[10] (see Table I)

TABLE I

STRUCTURES OF OVALBUMIN CARBOHYDRATE SIDE-CHAINS CONTAINING TERMINAL 2-ACETAMIDO-2-DEOXY-D-GLUCOSYL GROUPS



R = β-D-GlcNAc-(1→4)-β-D-GlcNAc-Asn

Fraction	*R*1	*R*2	*R*3	*Relative yield*[a]	*References*
1	α-D-Man-(1→6)	α-D-Man-(1→3)	β-D-Gal-(1→4)-β-D-GlcNAc-(1→4)	6.0	7
2	—	α-D-Man-(1→3)		10.7	7
3	α-D-Man-(1→6)	α-D-Man-(1→3)	β-D-GlcNAc-(1→4)	10.7	7
4	α-D-Man-(1→6)	α-D-Man-(1→3)	—	45.5	6,13
5	—	α-D-Man-(1→3)	β-D-GlcNAc-(1→4)	8.7	6
6[b]	β-D-GlcNAc-(1→6)	β-D-GlcNAc-(1→2)	β-D-Gal-(1→4)-β-D-GlcNAc-(1→4)	8.0	
7	—	α-D-Man-(1→3)	—	3.9	14
8	—	β-D-GlcNAc-(1→2)	β-D-GlcNAc-(1→4)	11.4	8,9,14

[a]The relative proportions of these fractions was obtained from their yields, by weight, after isolation from a digest of 100 g of pooled, native ovalbumin. Estimated accuracy is ±0.5. [b]Structure **6** has not been reported elsewhere; but its structure was tentatively inferred from its natural-abundance, ^{13}C-n.m.r. spectrum by comparison with those of other, known structures[10]. Structure **7** has the same carbohydrate composition as reported[14], but its structure was determined[10,13] by ^{13}C-n.m.r. spectroscopy and enzymic degradation from structure **4**.

linkage involved. One way in which to investigate this problem would be to isolate the individual glycopeptides generated from the D-Gal-labeled ovalbumin, and then to analyze their primary structures and their relative proportions, as was done previously for the unlabeled ovalbumin[5-8,10]. For the present study, we have isolated glycopeptide fractions as before, but have chosen a different analytical approach. We have analyzed the ^{13}C-n.m.r. spectra of the crude glycopeptide fractions of ovalbumin that had been labeled with ^{13}C-enriched D-Gal groups. The basic premise of this approach is that it should be possible to observe chemical-shift differences

for at least C-1 of Gal groups linked to the different β-D-GlcNAc residues. Such small differences in chemical shift have been observed[11] for the glycopeptides of asialo-α_1-acid glycoprotein using ^{1}H-n.m.r. spectroscopy. It would not be expected that the different anomeric linkages of the D-mannosyl residues would affect the chemical shifts observed for β-D-Gal resonances, as they are too far removed from each other. Thus, differentiation between β-D-GlcNAc-(1→2)-α-D-Man-(1→3)- and β-D-GlcNAc-(1→2)-α-D-Man-(1→6)- as acceptor sites would not be expected; it would be unlikely even in the case of β-D-GlcNAc-(1→4)-α-D-Man vs. β-D-GlcNAc-(1→4)-β-D-Man (for structures, see Table I).

In an earlier report[4], successful addition of D-Gal (20% uniformly enriched in ^{13}C) onto the carbohydrate side-chains of native, hen ovalbumin using D-galactosyltransferase* was described. The degree of incorporation varied from ~0.3 mol of D-Gal per mol of ovalbumin to ~0.6 mol/mol, depending on the preparation. Six new ^{13}C resonances, corresponding to a terminal β-D-Gal group, were observed in the spectrum of the modified ovalbumin[4]. The spectra were recorded at 25 MHz for relatively high concentrations of glycoprotein solution; as a result, the line-widths of the β-D-Gal resonances were broad (15 Hz for C-1) compared with the spectral resolution[4]. Under those conditions, chemical-shift differences between the carbon resonances of β-D-Gal groups linked to β-D-GlcNAc-(1→4) or β-D-GlcNAc-(1→2) would not have been detected unless the differences were greater than ~0.6 p.p.m. By obtaining the corresponding glycopeptides from the D-[U-^{13}C]Gal-ovalbumin, it was possible to obtain relatively simple, ^{13}C-n.m.r. spectra with narrower line-widths (~4–6 Hz), owing to removal of the peptide backbone. In addition, the ^{13}C-n.m.r. spectra of the glycopeptides were recorded at a higher field-strength (60.5 MHz), thus allowing a greater chemical-shift dispersion in frequency units.

Fig. 2 shows the ^{13}C-n.m.r. spectra obtained for fractions 2 and 3. Fraction 2 essentially contains only carbohydrate structures that possess at least one terminal, or penultimate, β-D-GlcNAc unit[5–10]. In its spectrum (see Fig. 2A), it is clear that there are two resonances in the anomeric region of the spectrum (~104 p.p.m.), indicative of two types of β-D-Gal residue. Because the enzyme is known to link the D-galactosyl group, *via* a β-(1→4) linkage, to D-GlcNAc, it was concluded that the two resonances must correspond to β-D-Gal (1→4)-linked to the β-D-GlcNAc-(1→4)- and the β-D-GlcNAc-(1→2)- branches. A resonance corresponding to β-D-Gal linked to a β-D-GlcNAc-(1→6)- branch** was not anticipated, as it is a minor component among the carbohydrate side-chains of ovalbumin. According to the data listed in Table I, the expected ratio of β-D-GlcNAc-(1→4):β-D-GlcNAc-(1→6) is ~20:1; the signal-to-noise ratio of the spectrum would not readily permit detection of the minor signal from the β-D-GlcNAc-(1→6) component. In the ^{13}C-n.m.r. spectrum of fraction 3 (see Fig. 2B) are observed the same two resonances arising from C-1 of

*The carbohydrate structure shown in ref. 4 is incorrectly drawn; it should, in fact, correspond to carbohydrate structure **4**, given in Table I.

**See footnote on p. 144.

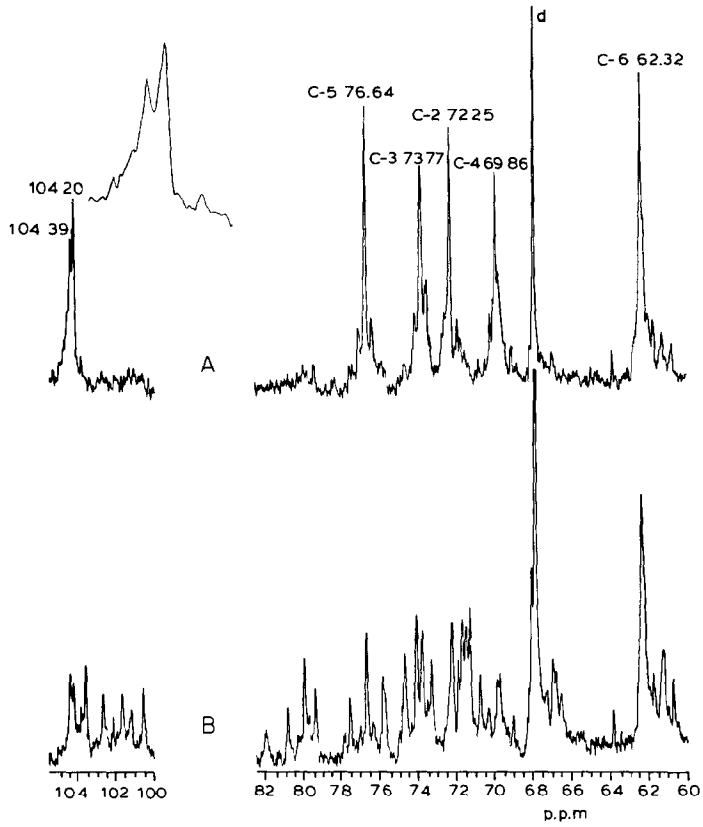


Fig. 2. A. The carbohydrate region of the ^{13}C-n.m.r. spectrum (60.5 MHz) of glycopeptide fraction 2. Only the β-D-Gal resonances are discernible in the spectrum. [The inset shows the anomeric-carbon region "splitting". No other such "splitting" could be discerned in the non-anomeric region.] B. The ^{13}C-n.m.r. spectrum of fraction 3, recorded under the same conditions. [Spectral conditions are given in the Experimental section. The 1,4-dioxane peak (labeled d) was used as the chemical-shift and intensity standard.]

the β-D-Gal groups, as well as signals arising from the high-D-mannose type of structures[10]. These peaks do not overlap with the β-D-Gal signals in the anomeric region[10,12].

The peak resonating at 104.20 p.p.m. is apparently due to C-1 of D-galactosyl groups linked to the β-D-GlcNAc-(1→4) branches, as precisely the same chemical shift ($\pm$0.03 p.p.m.) is observed for "native" β-D-Gal residues [linked only at the β-D-GlcNAc-(1→4) branch] in the natural-abundance, ^{13}C-n.m.r. spectra of three different, native, ovalbumin glycopeptides[10]. Thus, the other carbon resonance, at 104.39 p.p.m., must arise from C-1 of a β-D-Gal group linked to the β-D-GlcNAc-(1→2) branch. Only the C-1 resonance of the ^{13}C-enriched β-D-Gal groups displays observable, chemical-shift nonequivalence, due to the presence of β-D-GlcNAc residues having different glycoside linkages. In the non-anomeric region (80–60 p.p.m.),

the β-D-Gal resonances would be expected to overlap with the natural-abundance ^{13}C resonances of other residues in the structures, and this would affect both the intensity and degree of spectral resolution for those β-D-Gal resonances. Also, direct intensity or integration comparison for respective non-anomeric β-D-Gal resonances in Figs. 2A and 2B is not possible, as fractions 2 and 3 contain different carbohydrate structures, and thus, natural-abundance contributions are expected to be different in the two spectra. It may be noted that the chemical-shift position of C-1 of β-D-Gal-(1→4) is considerably downfield (~0.5 p.p.m.) of any other anomeric resonance found in the spectra of these fractions[10].

The relative distribution of D-galactose residues in the overall structures can be quantitatively estimated. For nonselective, random attachment of D-galactose, facilitated by the enzyme, the intensity ratio of the C-1 resonances in the *combined* fractions would be expected to reflect the original ratio between β-D-GlcNAc-(1→4) and β-D-GlcNAc-(1→2) residues in the native ovalbumin. There are, however, two assumptions imbedded in this expectation. First, the ratios between the glycopeptide structures isolated from ovalbumin reflect their real proportions in the intact glycoprotein. The second assumption is that the n.m.r. relaxation parameters for the two C-1 resonances are the same, or that the pulse-repetition period is large compared with the spin–lattice relaxation-time (T_1) for each. Within experimental error, it seems that these two approximations are valid. The first assumption cannot be directly proved, although, under the experimental conditions in which the preparation of the glycopeptides was conducted[5–8,10], very little selective hydrolysis of sugars from these structures would be expected. Because the pulse-repetition period is 0.7 s, and the T_1 value of the combined C-1 resonances at 25 MHz is[4] 0.14 s, the relaxation times for the two different C-1 atoms would need to be vastly different in order to invalidate the second assumption. The "native" β-D-Gal residues present at ^{13}C natural abundance in the sample also need to be accounted for. The calculated, ^{13}C-signal contribution from these β-D-Gal residues should be ~0.1% of the total signal-intensity observed, assuming that, proportionally, all available β-D-GlcNAc-(1→4) had been attached by ^{13}C-Gal.

From the ovalbumin glycopeptide-distribution[10], it is calculated that the enzyme can nonselectively transfer a maximum of ~1.0 mol of D-galactose per mol of ovalbumin. Neglecting contributions to the C-1 resonance of β-D-Gal from the "native" β-D-Gal residues, and *combining proportionally* the C-1 resonance intensities from the two fractions (2 and 3), it was found that the combined intensity-ratio for these two resonances (at 104.39 and 104.20 p.p.m., respectively) is 46:54 (±5). Integration of the combined, respective resonances yielded a similar ratio (49:51). From the relative proportions of the candidate structures (see Table I), and taking into account the existence of multiple receptor-sites for these structures, the ratio of β-D-GlcNAc-(1→2):β-D-GlcNAc-(1→4) receptor-sites is calculated to be 45:55 (±5). The ratio of resonance intensities for the *combined* fractions 2 and 3 is in excellent agreement with the β-D-GlcNAc-(1→2):β-D-GlcNAc-(1→4) ratio expected (based on the

structure distribution, see Table I); in fact, it is much better than experimental error would warrant.

This result should be critically examined. As the individual glycopeptide fractions were not isolated, and only 60% of the possible sites were utilized, there remains a possibility that the enzyme shows structural specificity toward some of the minor structures, or, alternatively, that some of the major glycopeptide components show some selectivity that is masked by the fact that the minor components do not show any selectivity. Although such possibilities cannot be ignored, it is considered that the good agreement between the experimental results and the calculated values tend to reinforce the foregoing conclusion. In particular, it may be pointed out that the major structures (**4** and **8** in Table I) contain equal proportions of β-D-GlcNAc-(1→4) and β-D-GlcNAc-(1→2) residues (57% of the total). It is also clear that the chicken D-galactosyltransferase does appear to exhibit distinct structural specificity, as the "native" attached D-galactose is found only on the β-D-GlcNAc-(1→4) units (structures **1**, **2**, and **6**); but, in this case, the total substituted-D-galactose sites constitute only a small fraction of the available sites (see Table I).

Thus, it is clear that bovine milk *N*-acetylglucosaminide-β-(1→4)-galactosyltransferase transfers D-Gal groups to both the β-D-GlcNAc-(1→4) and β-D-GlcNAc-(1→2) branches of native ovalbumin. With hen ovalbumin as the substrate, under conditions of saturation, the transfer of β-D-Gal groups is, most likely, made nonselectively to all possible sites, including the β-D-GlcNAc that is linked directly to the β-D-mannosyl residue in the core of the carbohydrate structures. This β-D-GlcNAc residue constitutes ~77% of the total β-D-GlcNAc-(1→4) residues in the mixture. Despite the fact that direct proof for D-Gal transfer to the β-D-GlcNAc-(1→6) branch was not obtained, the lack of enzyme specificity toward the other β-D-GlcNAc linkages suggests that this minor branch may also be terminated with β-D-Gal. Although there are several aspects of the carbohydrate side-chain that govern the ability of the enzyme to transfer D-galactose to the nonreducing terminus of the oligosaccharide, including a terminal β-D-GlcNAc and D-Man in the chain[3], the type of linkage from the terminal β-D-GlcNAc to the penultimate D-Man is apparently not a factor. Also, the overall structure of the side-chain in ovalbumin affects the kinetics of the transfer reaction[3], but it apparently does not induce enzyme selectivity toward the various terminal β-D-GlcNAc linkages present in these structures (see Table I). This may be contrasted with the finding that jack-bean α-D-mannosidase can show specificity toward the oligosaccharide structure remote from the site of cleavage in ovalbumin glycopeptides[10,13].

The ^{13}C-Gal labeling technique[4] was developed so that glycoproteins, in particular, membrane-surface glycoproteins, could be tagged with an innocuous n.m.r. probe of the oligosaccharide moiety, and subsequently used to study interactions with a variety of receptors (*e.g.*, lectins). The results of the present study imply that D-galactosyltransferase does not discriminate on the basis of GlcNAc glycoside linkage; this has obvious implications regarding studies of cell-surface glycoproteins *via* the ^{13}C-Gal labeling method.

We have also demonstrated that the use of ^{13}C-enriched sugars and ^{13}C-n.m.r. spectroscopy can help in structural analysis of sugar side-chains, without the need for larger samples (the total β-D-[U-^{13}C]-Gal in the sample being estimated to be ~5 mg).

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