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Hydrogen-Bonded Structure of the Complex N-Linked Fetuin Glycopeptide†

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ABSTRACT: The conformation of the N-linked complex glycopeptide of fetuin was examined with hydrogen-exchange techniques. The glycopeptide molecule contains eight acetamido hydrogens stemming from five *N*-acetylglucosamine residues and three *N*-acetylneuraminic acid residues and also one from the remaining sugar–peptide linkage. The hydrogen-exchange rates of these secondary amides were compared with small molecule model compounds having identical primary structures at their exchangeable hydrogen sites. Differences between the model rates and glycopeptide rates therefore cannot be accounted for by primary structure effects but reflect conformational features of the glycopeptide. Two glycopeptide hydrogens exhibit significantly hindered exchange; the rest exchange at the model rates. Removal of the three *N*-acetylneuraminic acid residues from terminal positions on the three branches of the glycopeptide removes the slowed hydrogens. The remaining ones continue to exchange at the model rate. These results indicate that two of the eight sugar acetamido hydrogens are involved in intramolecular hydrogen bonds. A likely structure includes two hydrogen bonds between the three *N*-acetylneuraminic acid residues. These two hydrogens, slowed to a moderate degree, reflect a preferred conformation stabilized by about 1 kcal/mol in free energy. The solution conformation of the glycopeptide suggested by these results is one that is partially ordered and can be easily modulated, owing to the relatively small amount of energy stabilizing the preferred conformation.

The structures of the oligosaccharide prosthetic groups of glycoproteins have become of great interest in recent years largely as a result of their probable role in cell-surface recognition phenomena (Ashwell & Morell, 1974; Hughes, 1976; Humphreys et al., 1977; Burger & Jumblatt, 1977; Lash & Burger, 1977; Frazier & Glaser, 1979). Knowledge of the primary structures of glycoprotein carbohydrate chains, aided by the development of new techniques for their determination (Spiro, 1973; Kornfeld & Kornfeld, 1976), has grown in concert with increasing interest in their function. Most chains can be categorized as one of two types: those attached to the polypeptide backbone by an O-glycosidic bond to serine or threonine, and those attached by an N-glycosidic bond to asparagine. The O-glycosidically linked chains are frequently the smaller of the two types and as a class exhibit fewer general

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structural similarities (Turco & Robbins, 1978). The larger N-glycosidically linked chains can be classified as either simple (also call high-mannose) or complex, depending upon their monosaccharide composition (Kornfeld & Kornfeld, 1976). The simple chains are composed of mannose and *N*-acetylglucosamine (GlcNAc)[1] residues alone; the complex chains contain, in addition, galactose, AcNeu, and sometimes fucose. The N-linked fetuin glycopeptide (Figure 1) dealt with in this work is an example of a complex chain.[2]

Most of the N-linked glycopeptide structures determined so far are derived from serum glycoproteins, and only recently have reports on the detailed primary structures of membrane glycopeptides of the complex N-linked-type appeared. The structures determined for a glycopeptide of fibronectin (Carter & Hakomori, 1979) and for a glycopeptide from a hamster cell membrane glycoprotein (Santer & Glick, 1979) show striking similarities to those of glycopeptides derived from serum glycoproteins. All exhibit a similar core structure (the sequence of sugars nearest the polypeptide attachment site) of $(GlcNAc)_2(mannose)_3$ and branches with the sequence GlcNAc-galactose-AcNeu (see Figure 1). The major difference among them is in the number of peripheral branches attached to the core, usually two or three. Minor differences include a few variations in the linkage positions, the presence or absence of AcNeu residues at the nonreducing ends, and the frequent presence of a fucose residue attached to a core GlcNAc residue. A more general structural analysis of serum glycopeptides and a comparison of these with glycopeptides from a variety of tissues further corroborate the observation that the general structures of N-linked glycopeptides fall into just a few classes with the major difference between classes being in the number of peripheral branches (Finne & Krusius, 1979). Further, while the fetuin-type glycopeptide is representative of roughly one-fourth of the population of serum glycopeptides, this triantennary type of chain is even more characteristic of tissue glycopeptides.

In contrast with the growing knowledge of primary structures, knowledge of possible higher order structures is quite limited. Preferred conformations have been described for some periodic polysaccharides (e.g., xanthan, agarose, and carrageenans) in the condensed state, and the evidence suggests that in some cases these conformations survive under appropriate solution conditions (Rees, 1975; Morris et al., 1977). A proton nuclear magnetic resonance study of glycosaminoglycans in solution (Welti et al., 1979) indicated the presence of tenuous interactions in hyaluronate, while no ordered solution conformation has been observed for any of the sulfated glycosaminoglycans (Lindahl & Hook, 1978). Carbon-13 nuclear magnetic resonance techniques were applied (Sillerud et al., 1978) to ganglioside G_{M1}, a molecule with head-group primary structure similar to glycoprotein sugar side chains, and used to construct a model for the cation binding site based on perturbations induced by paramagnetic europium(III). Magnetic resonance and circular dichroism studies carried out on the compound GlcNAc-asparagine, a molecule representing the linkage region of the N-linked complex-type glycopeptides, were interpreted in terms of a model for the conformation of the linkage region (Bush et al., 1980). Conformations of small oligosaccharides representing portions of blood-group determinants were predicted by hard-sphere calculation, taking into account the exo-anomeric effect (Lemieux et al., 1980). Solution conformational parameters determined for these molecules by nuclear magnetic resonance techniques appear to agree to a large extent with those calculated and certainly indicate the existence of preferred glycosidic bond angles. Finally, crystallographic studies on an immunoglobulin G (IgG) molecule showed that under certain conditions (perhaps analogous to antigen binding) the complex carbohydrate chain linked to the F_c region adopted a stable conformation (Huber et al., 1976). This important result indicates that N-linked complex heteropolysaccharide chains can adopt ordered conformations.

The paucity of information concerning the shapes and interactions of the oligosaccharide chains of glycoproteins, coupled with the growing conviction that these same moieties are primary effectors in many fundamental biological processes, has prompted us to probe the structure of the N-linked complex glycopeptide of the serum glycoprotein fetuin (Figure 1). As pointed out above, the structure of this glycopeptide is quite representative of those derived from the cell surface as well as those from serum glycopeptides.

Hydrogen-exchange techniques appear well suited to our purposes even though applications thus far have been limited to structural issues in proteins and nucleic acids (Englander & Englander, 1978; Englander et al., 1972; Woodward & Hilton, 1979). Rates observed for the exchange of peptide hydrogens in proteins, for example, can range over 8 orders of magnitude. These variations in exchange rate reflect features of macromolecular conformation and stability. In the fetuin complex glycopeptide, there are nine amide hydrogens scattered through the molecule. Our results show that while seven of these hydrogens exchange at a rate indicating free contact with solvent, two hydrogens exchange at a significantly slowed rate, implying their involvement in intramolecular hydrogen bonds.

Materials and Methods

Materials. *N*-Acetyl-D-glucosamine (GlcNAc), *N*-acetylneuraminic acid (AcNeu), and powdered chitin (practical grade) were all purchased from Sigma. Fetuin, prepared according to the method of Spiro (1960), was obtained from Grand Island Biological Co. Pronase (B grade) was from Calbiochem, Darco G-60 charcoal was from Matheson Coleman and Bell, and Celite 535 was from Johns-Manville. D_2O (99.8 mol %) was purchased from Bio-Rad. All other chemicals used were reagent grade or better.

Preparation of $(GlcNAc)_3$. The β1–4-linked trimer of GlcNAc was isolated by acid hydrolysis of chitin and fractionation of the resulting products on a charcoal/Celite column eluted with an ethanol/water gradient, as described by Rupley (1964). Fractions from the charcoal column containing the trimer were pooled, lyophilized, and subjected to gel filtration (Sephadex G-25) using 10 mM ammonium acetate in 20% ethanol. Contaminating species, largely the dimer and tetramer, appeared as small shoulders on the $(GlcNAc)_3$ peak and were discarded. The resulting preparation of $(GlcNAc)_3$ appeared homogeneous by paper chromatography according to Powning & Irzykiewicz (1965) and comigrated with a standard sample of $(GlcNAc)_3$ generously supplied by Dr. B. Sykes.

Preparation of N-Linked Fetuin Glycopeptide. GIBCO fetuin (1 g), used without further purification, was digested

[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine; AcNeu, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; $(GlcNAc)_3$, the β1–4 glycosidically linked trimer of GlcNAc; HTX buffer, hydrogen–tritium-exchange buffer (see Materials and Methods); pD_r, the value read on the pH meter when the glass electrode is inserted into a buffered D_2O solution.

[2] The term glycopeptide refers to the oligosaccharide chain of a glycoprotein containing a minimum number of amino acid residues around the attachment site, the result of extensive proteolytic digestion of the glycoprotein.

with Pronase as described by Spiro & Bhoyroo (1974) except that the amount of Pronase initially added corresponded to 2% of the weight of the fetuin rather than 1%. After lyophilization, the digested material was applied to a Sephadex G-25 column measuring 2.5 × 82 cm and eluted with 10 mM ammonium acetate in 20% ethanol. Glycopeptide elution was followed by using the phenol/sulfuric acid neutral carbohydrate assay (Dubois et al., 1956) scaled to smaller volumes. The glycopeptide peak emerged slightly after the void volume and exhibited a shoulder on the low molecular weight side, indicating partial resolution of the N-glycosidically linked oligosaccharide from the smaller O-glycosidically linked oligosaccharide (Spiro & Bhoyroo, 1974; Baenziger & Fiete, 1979). Shoulder fractions were discarded. The peak fractions were pooled, lyophilized, and again subjected to Pronase digestion. The resulting mixture was again filtered through Sephadex G-25, and elution was monitored by the thiobarbituric acid assay for sialic acids (Warren, 1959) after removal of the AcNeu by hydrolysis in 0.1 N sulfuric acid at 80 °C for 1 h (Spiro, 1966), as well as by the neutral hexose assay. The glycopeptide eluted slightly after the void volume, and separation from the digested peptide material was confirmed by assaying fractions for peptide using the fluorescamine primary amine method (Lai, 1977). The ratio of absorbance in the AcNeu assay to absorbance in the neutral hexose assay was constant across most of the peak but increased on the trailing side, indicating the presence of contaminating O-linked glycopeptides. The fractions thus identified were discarded. Remaining fractions were pooled and lyophilized, and the dry, purified N-linked glycopeptides were stored at −20 °C.

Characterization of Glycopeptide. The purified glycopeptide was assayed quantitatively by both the thiobarbituric acid assay and the phenol/sulfuric acid neutral hexose assay. AcNeu was cleaved from the glycopeptide before the thiobarbituric acid assay by mild acid hydrolysis in 0.1 N sulfuric acid at 80 °C for 1 h. Standard curves were obtained by using AcNeu for the thiobarbituric acid assay and galactose and mannose for the neutral hexose assay.

Amino acid analyses of the purified glycopeptide were performed on an automated Durrum analyzer after hydrolysis in 6 N hydrochloric acid at a 110 °C for 24 h in evacuated glass bulbs. GlcNAc and GalNAc (as the free amino sugars) were also measured on the amino acid analyzer after hydrolysis in 4 N hydrochloric acid at 110 °C for 5.5 h (Spiro, 1972).

Determination of the molecular weight of the glycopeptide was carried out by the short-column sedimentation equilibrium method (Yphantis, 1960), using a 3-mm column, with a Beckman Model E ultracentrifuge at 15 220 rpm and 22 °C. For these experiments, the glycopeptide was dissolved at 1.6% (w/v) in the same buffer as was used for the hydrogen–tritium-exchange experiments.

Preparation of Asialoglycopeptide. Terminal AcNeu was removed from the glycopeptide by hydrolysis with 0.075 N sulfuric acid at 80 °C for 1 h (Spiro, 1966). Liberated AcNeu was separated from the asialoglycopeptide by filtration on Sephadex G-25 in 10 mM ammonium acetate in 20% ethanol. Further incubation of an aliquot of the asialoglycopeptide in 0.1 N sulfuric acid at 80 °C for 1 h resulted in no further release of AcNeu.

Hydrogen–Deuterium Exchange by Ultraviolet Spectrophotometry. The hydrogen–deuterium-exchange behavior of AcNeu was measured spectrophotometrically as recently described (Englander et al., 1979). Briefly, a small volume (50 or 75 μL) of aqueous buffer containing the sugar was diluted into 1 mL of a similarly buffered D_2O solution at 15 °C in a standard quartz cuvette in the thermostated sample chamber of a Cary 118 spectrophotometer. The decrease in the absorbance at 220 nm—a result of the replacement of the acetamido protons with deuterons—was recorded as a function of time and computer fit to obtain the exchange rate constants. For experiments in the pH range below 3.5, glycine was used as a buffer, in the pH range 3.5—5.5 acetate was used, and cacodylate was used above pH 5.5. The composition of the solution (HTX buffer) was 10 mM appropriate buffer, 0.1 M sodium chloride, 5 mM calcium chloride, 3.5 mM potassium chloride, and 0.5 mM magnesium chloride. The sugar used had an absorbance of 0.8–1.0 unit, and the exchange-dependent change in absorbance amounted to 5% of this value. Immediately after the exchange experiment, a pH meter reading was recorded as pD_r (pD_{read}).

Hydrogen–Tritium Exchange by Gel Filtration. The tritium-exchange behaviors of $(GlcNAc)_3$, fetuin glycopeptide, and fetuin asialoglycopeptide were measured by using the Sephadex column method (Englander & Englander, 1972). All tritium-exchange experiments were carried out at 0 °C and pH 5.50 in HTX buffer using Sephadex G-10 to separate bound from free tritium. Exchange-in mixtures for the glycopeptides were prepared at a concentration of 16 or 4 mg/mL while mixtures for $(GlcNAc)_3$ were prepared at 5 mg/mL. The macromolecular eluates from the columns separating the labeled macromolecule from released tritium were collected in several tubes, and the counts/absorbance (C/A) ratio was determined separately for each fraction. The concentrations listed allowed the collection of several fractions which after appropriate dilution yielded absorbance readings in the 0.3–0.6 range.

The number of hydrogens remaining per molecule (H/M) is calculated according to $H/M = [(111C/C_0)/(A/\epsilon)]/1.19 = [(111\epsilon/C_0)(C/A)]/1.19$, where C_0 is the number of counts in the exchange-in mixture, A/ϵ and C are the molar concentrations of macromolecule and counts, respectively, after exchange-out, and 111 is the gram-atom concentration of hydrogen in water (Englander & Englander, 1972). The factor of 1.19 is included to correct for the tritium–hydrogen equilibrium isotope effect for secondary amides (Englander & Poulsen, 1969). ϵ_{220} for $(GlcNAc)_3$ was found to be 347 M^{-1} by use of the ferricyanide reducing sugar assay (Dische, 1962). ϵ_{225} values for the glycopeptide and asialoglycopeptide were 2648 and 1927 M^{-1}, respectively, as determined by the thiobarbituric acid and neutral carbohydrate assays as previously described. The difference between these values matches to within a few percent 3 times the extinction coefficient for AcNeu.

Results

Purity of Glycopeptide. The identity of the N-linked glycopeptide as well as its separation from digested peptide material and the smaller O-linked glycopeptides was documented by several independent results.

(1) Glycopeptide composition assays for AcNeu and neutral carbohydrate agreed to within 4% when it was assumed that 1 mol of glycopeptide contains 3 mol each of AcNeu, mannose, and galactose. Similarly, amino sugar analyses indicated 5 mol of GlcNAc per mol of glycopeptide. These results are all consistent with the structure in Figure 1.

(2) The level of contamination of the isolated product by O-glycosidically linked glycopeptides can be inferred from the amount of GalNAc remaining (Spiro & Bhoyroo, 1974), as this residue is present only in the smaller species. We found less than 0.1 mol of galactosamine per mol of N-linked gly-

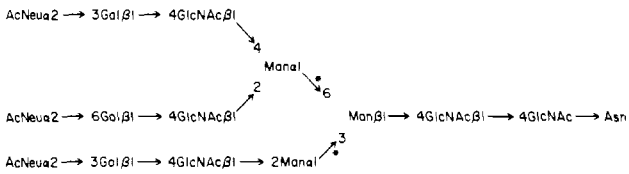

FIGURE 1: Structure of the N-linked fetuin glycopeptide. The structure shown is that proposed by Baenziger & Fiete (1979). A slightly different structure which interchanges the positions of the two linkages marked with asterisks has been proposed by Nilsson et al. (1979). The sugar abbreviations are AcNeu, *N*-acetylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetyl-D-glucosamine; and Man, mannose.

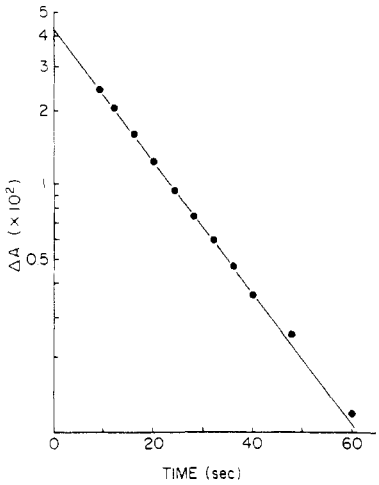

FIGURE 2: Hydrogen–deuterium exchange of AcNeu measured by the ultraviolet spectrophotometric method at 15 °C and pD_r 5.83. The linear semilogarithmic plot measured over four half-times indicates the first-order nature of the process under these conditions.

copeptide (i.e., per 5 mol of GlcNAc). The O-linked glycopeptide contains 1 mol of GalNAc per mol of glycopeptide; therefore, the preparation contains, on a molar basis, less than 10% of the O-linked glycopeptide.

(3) The purified sample contained 1 mol of aspartate (from asparagine, the linkage amino acid residue), 0.3 mol of serine, 0.3 mol of glycine, and traces of threonine, glutamate, and alanine. This rules out significant contamination by peptide material and indicates that, on the average, each glycopeptide molecule contains less than one amino acid residue in addition to the linkage asparagine.

(4) The molecular weight determined by sedimentation equilibrium was 3030, compared with the value of 2990 calculated for the molecule shown in Figure 1 (including one asparagine). For this determination, we used $\bar{V} = 0.653$ mL/g, calculated from Spiro's value of 0.664 mL/g (Spiro, 1962) for his fetuin glycopeptide preparation which contained 23% peptide (assumed peptide $\bar{V} = 0.70$). This result, in addition to supporting the purity of the preparation, also rules out significant aggregation as a possible explanation for the slowed hydrogens.

Exchange Rates for Model Compounds. Rates of exchange of the acetamido hydrogens with solvent hydrogens were measured for AcNeu by ultraviolet spectrophotometry and for $(GlcNAc)_3$ by the tritium–Sephadex method. Figure 2 is a semilogarithmic plot showing the absorbance decrease resulting from the exchange of protons for deuterons when AcNeu was diluted into D_2O buffer at $pD_r = 5.83$. The exchange rates obtained from a number of such experiments at various pD_r values are plotted on a logarithmic scale as a function of pD_r in Figure 3. The solid line, drawn with unit slope, indicates catalysis of the exchange by OH^- ion above pD_r 4.5; this has been observed for all amide groups studied. The deviation at

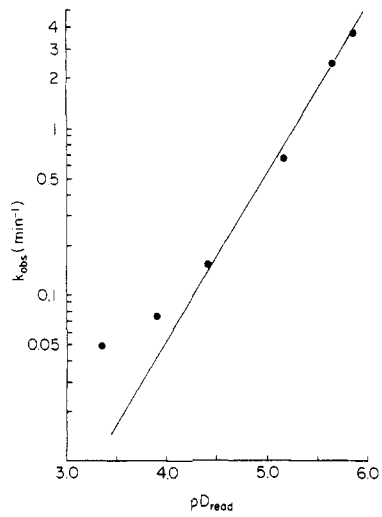

FIGURE 3: Log–log plot of AcNeu exchange rate constants as a function of pD_r at 15 °C. The solid line is drawn with a slope of 1 as required by theory for catalysis by the hydroxyl ion. Deviation at low pH indicates H^+ and/or H_2O catalysis.

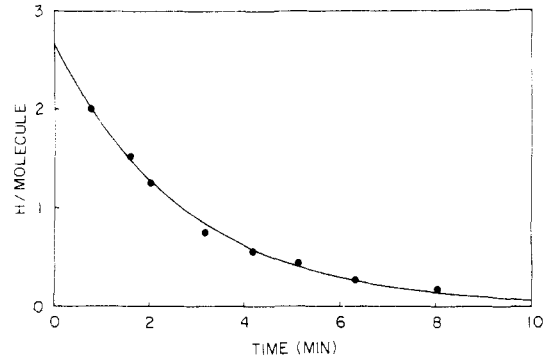

FIGURE 4: Hydrogen–tritium exchange of $(GlcNAc)_3$ at 0 °C and pH 5.50. The curve drawn is the fit for a single exponential decay with an amplitude of 2.7 hydrogens, and a rate constant of 0.37 min^{-1}.

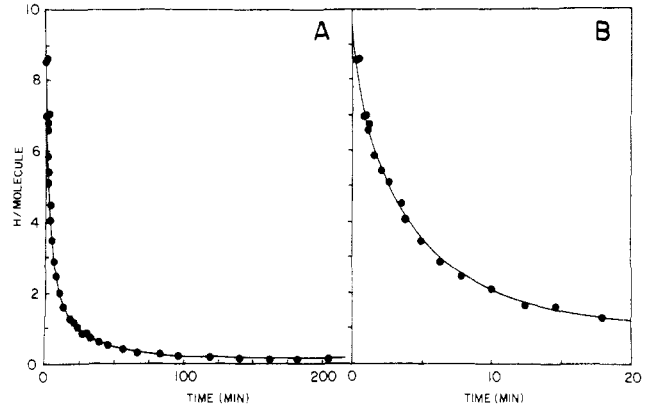

FIGURE 5: Hydrogen–tritium exchange of fetuin glycopeptide at 0 °C and pH 5.50. The curve drawn is the plot of the best-fit function which is a triple exponential decay above a background of 0.173 (1.6%). The amplitudes and rate constants, respectively, for the three kinetic classes are as follows: ~2.5 H per molecule, 2.0 min^{-1}; 5.8 H per molecule, 0.25 min^{-1}; and 2.1 H per molecule, 0.038 min^{-1}. (A) Data and theoretical curve plotted on a time scale which includes data points at longest times measured. (B) Data and theoretical curve plotted only up to 20 min to show short time points.

lower pH indicates catalysis by H^+ or H_2O.

Figure 4 shows hydrogen–tritium-exchange results for $(GlcNAc)_3$ using the Sephadex column method. The data describe a single exponential decay, indicating that all three of the acetamido hydrogens exchange with similar rates. The

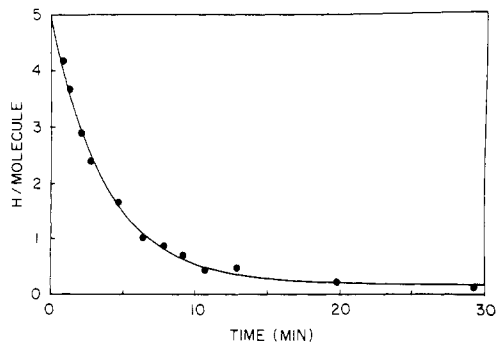

FIGURE 6: Hydrogen–tritium exchange on desialylated fetuin glycopeptide at 0 °C and pH 5.50. The best-fit curve shown is a single exponential decay above a background of 0.21 (4%). The fit indicates 4.8 hydrogens per molecule with a rate constant of 0.27 min^{-1}.

number of exchanging hydrogens measured is 2.7, compared to the expected value of 3 for $(GlcNAc)_3$. Exchange experiments were also carried out on the GlcNAc monomer by the spectrophotometric method exactly as described for AcNeu. The results of these experiments, while more complicated, suggest exchange rates for GlcNAc very close to those determined for AcNeu.[3]

Exchange Rates for Fetuin Glycopeptide. The data from hydrogen-exchange experiments on the fetuin glycopeptide by the tritium–Sephadex method are shown in Figure 5. The data were fit well by three exponentials. The best-fit parameters are given in the figure legend; they indicate about two, six, and two hydrogens in the three kinetic classes. The glycopeptide contains nine to ten secondary amides—five from GlcNAc residues, three from AcNeu residues, one from the GlcNAc–asparagine N-glycosidic linkage, and a fraction of one from peptide bonds joining residual amino acids to asparagine. The exchange rate exhibited by each class differs by about 7-fold from the rate of the adjacent class. Several exchange experiments performed in HTX buffer without Ca^{2+}, Mg^{2+}, or K^+ yielded results indistinguishable from these.

Hydrogen–tritium-exchange experiments were also carried out on the desialylated glycopeptide under conditions identical with those used for the intact glycopeptide. Figure 6 shows the results of these experiments. In contrast, these data are fit well by a single exponential; the best-fit parameters are given in the legend. The number of exchangeable hydrogens in this case is close to five (4.8 best fit). The rate observed for these protons (0.27 min^{-1}) is very close to that found for the analogous class in the intact glycopeptide (0.25 min^{-1}) which numbers 6 (Table I summarizes rate data).

Table I: Summary of Hydrogen-Exchange Results for Fetuin Glycopeptides and Model Compounds[a]

Models	
AcNeu	0.34 (1)
$(GlcNAc)_3$	0.37 (3)

	Glycopeptide	
kinetic class	intact glycopeptide	desialylated glycopeptide
fast	2.0 (2)	NM
medium	0.25 (6)	0.27 (5)
slow	0.038 (2)	–

[a] Exchange rates, given in units of $(minutes)^{-1}$ refer to pH 5.5 and 0 °C. Rates are thought to be accurate to ±10%. The number of hydrogens in each kinetic class is shown in parentheses. (Exact numbers found in the computer fits are included in the figure legends.) The rate shown for the model monosaccharide AcNeu is the result of division of the rate measured at 15 °C by a factor of 5.16 to correct to 0 °C (see text). NM = not measured, since data points earlier than 1 min were not taken. (–) Removal of the three terminal AcNeu residues results in the loss of both of the slowed acetamido hydrogens and one of the exposed actamido hydrogens.

Suitability of Model Compounds. AcNeu and $(GlcNAc)_3$ are proper models with which to compare the exchange behavior of the glycopeptides, since the relevant portions of their chemical structures match the corresponding structures in the glycopeptide. In the absence of secondary structure, the monosaccharide AcNeu should exhibit acetamido hydrogen-exchange rates like those for a C-2-linked AcNeu residue, since the secondary amide group is attached several bonds away from the carbon atom involved in the linkage, and should therefore be minimally affected by the linkage (Molday et al., 1972). The acetamido group in the glycopeptide GlcNAc, however, is adjacent to the carbon atom involved in the glycosidic linkage and therefore may be expected to exhibit different hydrogen-exchange rates depending upon the linkage status of C-1. An appropriate model for the GlcNAc residues in the glycopeptide is $(GlcNAc)_3$, whose β1–4 glycosidic linkages are the same as those flanking GlcNAc residues in the glycopeptide.

The comparison between model exchange rates and glycopeptide exchange rates, in the case of $(GlcNAc)_3$, is direct since both were evaluated with hydrogen–tritium techniques under identical buffer conditions at 0 °C. The exchange data from AcNeu, however, were obtained in hydrogen–deuterium-exchange experiments at 15 °C, so that temperature dependence and isotope effects must be taken into account. The tritium-exchange experiments were carried out at pH 5.50, a pH at which the exchange is clearly base catalyzed (see Figure 3). Accurate measurements of the temperature dependence of the base-catalyzed reaction indicate an activation energy of 17.4 kcal mol^{-1} (Englander et al., 1979), so that the rate obtained at 15 °C should be lowered by a factor of 5.16 to obtain the rate at 0 °C. Study of the relevant isotope effects (Englander et al., 1979) has shown that the exchange rate for a secondary amide determined from a hydrogen–deuterium experiment (in D_2O) is essentially identical with the rate determined from a hydrogen–tritium experiment (in H_2O) when pD_r is numerically equal to pH. We can therefore compare directly the results of the two kinds of experiments.

Adjusted in the manner just described, our data indicate the rate of exchange of the acetamido hydrogen of AcNeu at

[3] At certain pD_r values, the exchange curve for GlcNAc is biphasic, indicating a process more complex than simple exchange from a single species. In the experiments in which the curve was fit best with two exponentials, the relative sizes of the classes were 2:1. This matches the relative populations of the α and β anomers in solution (Horton et al., 1966), suggesting that the more complex kinetics may be a result of different exchange rates for the two GlcNAc anomers. At more basic pD_r values, the deviation from single-class kinetics is much less obvious, and at least 90% of the absorbance change is accounted for by a single exponential decay. Both hydrogen exchange and mutarotation are acid and base catalyzed, and the rates may become comparable in a part of the pH range considered here (Pigman & Isbell, 1968; Isbell & Pigman, 1969). Thus, it is not surprising that strict single-class kinetics are not observed. These results, while making it difficult to obtain an accurate rate constant for GlcNAc acetamido proton exchange, do not jeopardize the validity of our interpretation of the glycopeptide exchange since the two GlcNAc rates differ only by about 3-fold and are very similar to the rate for $(GlcNAc)_3$, and since the correct exchange behavior comparison is made between the glycopeptide and $(GlcNAc)_3$. Both of these molecules contain β1–4 glycosidic linkages adjacent to C-2, which contains the acetamido group. The linkage prevents mutarotation, thereby "locking" all GlcNAc's in the glycopeptide in the β form. In $(GlcNAc)_3$, one of the three sugars is free; thus, mutarotation can occur, giving rise to the α and β anomers. Nevertheless, the tritium-exchange results on $(GlcNAc)_3$ at 0 °C and pH 5.50 are dominated by a single first-order class (see Figure 4 and text).

0 °C and pH 5.50 to be 0.34 min^{-1}. The exchange rate for the acetamido hydrogens of $(GlcNAc)_3$, determined under these conditions (Figure 4), is 0.37 min^{-1}. The fact that the rates for these two molecules are essentially identical is not surprising when considered in light of their structural homology around the exchangeable hydrogen site. Given the exchange rates in the proper small molecule models, we can now consider their exchange rates in the glycopeptide, confident that any differences observed are the result of conformational features rather than primary structure effects.

Glycopeptide Exchange Is Different from That of Model Compounds. The exchange behavior of the fetuin glycopeptide (Figure 5) does not represent the simple sum of its constituent sugars; that is, the exchange curve is not a single exponential with a rate constant of about 0.35 min^{-1}. In fact, three kinetic classes are observed. The largest class, composed of approximately six hydrogens, exchanges at a rate (0.25 min^{-1}) close to that found for the small molecule models, indicating that six of the glycopeptide's amide hydrogens are about as readily available for exchange as the amide hydrogens in the free sugars. The exchange rate of these exposed hydrogens is less than the free model rate by a factor of 1.3. The reason for this very small difference is not clear, and we shall use the slower rate as the glycopeptide exposed rate. Two amide hydrogens exhibit an exchange rate approximately 7-fold slower than the exposed rate, indicating that their exchange is hindered relative to that of the other amide hydrogens. A small faster class (about two hydrogens) appears to represent the carbohydrate–asparagine linkage amide proton plus residual peptide moieties. The amide formed from the linkage of the innermost GlcNAc residue to the asparagine is one for which we have no model compound. The surrounding chemical structure, however, is similar to the structure around peptide groups in proteins. The rates characteristic of secondary amides so disposed have been measured by Molday et al. (1972), and the rate of about 2 min^{-1} we have measured here for the fast class is as expected for an amide with the structure of the linkage amide in GlcNAc-Asn. Likewise, a similar rate would be expected for peptides joining any other remaining amino acid residues to the linkage asparagine. Since the amino acid analysis data show that there remains, on the average, about two-thirds of an additional amino acid residue, the total number of hydrogens exchanging at the faster rate would be close to two. Thus, our assumption of the linkage amide and a peptide group as the sources of the rapidly exchanging class of hydrogens seems quite reasonable. The six exposed hydrogens and the two slowed hydrogens are then assigned to the eight acetamido groups of the GlcNAc and AcNeu residues.

Removal of the three AcNeu residues from the glycopeptide results in the disappearance of the two slowed hydrogens and one hydrogen of the intermediate rate class. The resulting desialylated glycopeptide (Figure 6) exhibits a single kinetic class of five exchanging hydrogens, equal to the number of acetamido groups remaining (all GlcNAc). The exchange rate of these hydrogens (0.27 min^{-1}) matches the rate found for six of the hydrogens (0.25 min^{-1}) in the intact glycopeptide. The small fast class cannot be seen in Figure 6 owing to the absence of data points in the hard to measure early time region.

Discussion

Six of the eight amino sugar acetamido hydrogens in the N-linked fetuin glycopeptide exchange at close to the model rate; two are 7-fold slower than these (Table I). The conformational features responsible for the slowing of hydrogen-exchange rates include both physical inaccessibility of the exchangeable hydrogen to solvent and hydrogen bonding. It has been argued (Englander et al., 1980) that in practice the important factor is hydrogen bonding and that the slowing of a hydrogen-exchange rate is an indication of the involvement of the exchangeable hydrogen in a hydrogen bond. Others have argued for a more important role for the physical exclusion of solvent from the exchangeable hydrogen site (Woodward & Hilton, 1979). In the case of the glycopeptide, it is difficult to imagine that water can be excluded from a non-H-bonded site since the molecule is small, not highly structured, and nearly uniformly polar. We conclude, therefore, that in the intact glycopeptide two of the eight sugar acetamido hydrogens are involved in intramolecular hydrogen bonds. Intramolecular rather than intermolecular interactions are indicated since 4-fold changes in the glycopeptide concentration had no effect on the hydrogen-exchange results, and sedimentation equilibrium experiments at similar concentrations of glycopeptide indicated no aggregation. It should not be overlooked that the rate of exchange of the five GlcNAc acetamido hydrogens in the desialylated glycopeptide, like the rate for six of the acetamido hydrogens in the intact glycopeptide, is similar to the rate found for the free model compounds (Table I). Again, this correspondence indicates that these hydrogens are readily available for exchange and not substantially hindered by virtue of their disposition in the macromolecule.

It is most reasonable to hypothesize that the GlcNAc acetamido hydrogens remain exposed and normally exchanging in the intact glycopeptide and that the slowed hydrogens represent two of the three AcNeu acetamido hydrogens. If this were not true, then attachment of the AcNeu residues (to the desialylated molecule) would have to alter the conformation some distance away, including the introduction of hydrogen bonds involving GlcNAc acetamido hydrogens, while still allowing for the free exchange of the AcNeu hydrogens themselves. Manipulation of a CPK glycopeptide model in an effort to create such a situation is difficult. On the other hand, the three branches of the molecule, when fully extended, naturally form two hydrogen bonds between the three terminal AcNeu residues. The appropriate alignment of the residues allows the middle AcNeu to serve as both hydrogen-bond donor (acetamido hydrogen) and hydrogen-bond acceptor (carboxylate group), while each outer AcNeu residue supplies either one acetamido donor or one carboxylate acceptor. This structure utilizes the strongest H-bond acceptors in the molecule, the electronegative carboxylate groups, minimizes steric constraints, leaves six of the acetamido hydrogens free for exchange as was observed, and of course explains the loss of the two slow hydrogens on desialylation. Although we feel this explanation to be the most likely one, others cannot be excluded. Evidence for long-range effects of AcNeu removal from oligosaccharides has been provided by nuclear magnetic resonance studies (Dorland et al., 1978), although the nature of such effects is obscure.

Certainly, the formation of these hydrogen bonds results in the loss of some conformational freedom. Can the decrease in conformational entropy be overcome by the preferential hydrogen-bonding energy? Study of a space-filling glycopeptide model suggests that the loss in conformational freedom is not large. The three chains, even in the absence of such a preferred conformation, have neither full freedom of rotation about their glycosidic bonds nor total independence in their overall rotation or translation. Rotation about the glycosidic bonds is severely restricted by the presence of bulky acetamido groups in equatorial disposition on the position-two carbons

of the GlcNAc residues. Independent motions and kinking of the three chains are similarly restricted by virtue of their mutual covalent attachment in the core region of the molecule. Therefore, the extended alignment of the three chains necessary to allow the formation of the hydrogen bonds appears to result in only a small further conformational restriction of a molecule already significantly limited in its internal motions.

The magnitude of the free energy stabilizing the preferred conformation (whether the one suggested or not) can be estimated if one assumes a simple two-state equilibrium involving the presence or absence of the two hydrogen bonds (Englander et al., 1972). The 7-fold slowing of the exchange rates indicates that each hydrogen bond is present six-sevenths of the time. This corresponds to a stabilization free energy of about 1 kcal/mol ($\Delta G = -RT \ln K$).

While we have accounted for and measured the exchange rates of all the secondary amides in the molecule, our data provide no direct information concerning the status of the hydroxyl hydrogens. Since the rate of exchange of free hydroxyl hydrogens is so much faster than that for secondary amides, they could be slowed substantially and still be too fast to be observed using our techniques. Therefore, the preferred conformation involving hydrogen bonding of two of the acetamido hydrogens may or may not involve hydrogen bonding of hydroxyl hydrogens as well. We can only rule out the presence of hydroxyl hydrogen bonds in any structure stabilized by more than about 4 or 5 kcal/mol in free energy. The exchange of hydroxyl hydrogens thus disposed would be observable in our results.

The net stabilizing energy of 1 kcal/mol indicates that the preferred conformation is a labile one. While this result is quite different from those for globular proteins, it is not without precedent. Similar results have been reported in experiments dealing with the hydrogen-exchange behavior of small peptide hormones (Krishna et al., 1979). These molecules have in common their function as recognition and signal entities participating in the molecular regulation of many biological processes. Their small energy of stabilization ensures that these molecules will adopt their preferred conformations most of the time and thus be available for recognition with high efficiency but still allows these molecules high conformational malleability, which may be valuable in their receptor interactions.

The complex N-linked fetuin glycopeptide was selected for this study because its primary structure exhibits the major characteristics of cell-surface glycoprotein carbohydrate side chains. It is reasonable to suspect, therefore, that the aspects of higher order structure implied by our results may be relevant to the complex sugar chains of the cell surface.

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Vesicle–Vesicle Interactions in Sonicated Dispersions of Dipalmitoylphosphatidylcholine†

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ABSTRACT: The time course of the size transformation of sonicated small unilamellar dipalmitoylphosphatidylcholine vesicles at 23 °C has been followed with ^{31}P and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Comparison of these results with turbidity measurements indicates that vesicle aggregation, monitored by turbidity, and size transformation, followed by NMR, occur on distinctly different time scales. For lipid concentrations in the 5–50 mM range, aggregation takes place on a time scale of minutes, whereas size transformation takes many hours. Aggregation, unlike size transformation, can be reversed by increasing the temperature above the phospholipid phase transition temperature. Analysis of the ^{31}P NMR line shapes provides evidence for a model in which the small vesicles transform into the product vesicles [characterized by Schullery, S. E., Schmidt, C. F., Felgner, P., Tillack, T. W., & Thompson, T. E. (1980) *Biochemistry 19*, 3919] without producing significant amounts of intermediate-size vesicles. Kinetic analysis indicates that the size transformation is apparently second order. ^{1}H NMR data indicate that the rate of transformation is decreased if trivalent ions are added to the dispersions and also if the temperature is periodically increased above the transition temperature. Analysis of the latter experiment provides some evidence that vesicle aggregation is a necessary precursor to size transformation. It was also found that increasing the average vesicle size decreases the extent of transformation.

Ultrasonic irradiation of phospholipid dispersions produces small unilamellar vesicles, 200–300 Å in diameter (Huang, 1969), which have been used in a variety of physical and biological studies (Papahadjopoulos, 1978). When the vesicles have been prepared from saturated fatty acid phosphatidylcholines, which undergo thermotropic phase transitions above 0 °C, there have been conflicting reports about their stability. Recently, however, it has become clear that if the phosphatidylcholines have been shown by heavy thin-layer chromatography spotting to be free of contaminants, then the small vesicles transform into larger species at an appreciable rate only below the gel to liquid-crystalline transition temperature, but not at or above it (Suurkuusk et al., 1976; Kantor et al., 1977; Larrabee, 1979; Schullery et al., 1980). This study, using ^{31}P and ^{1}H nuclear magnetic resonance (NMR) to follow changes in the size distribution of dipalmitoylphosphatidylcholine vesicles, was undertaken in an attempt to learn more about the mechanism of transformation, in the hope that this information will be applicable to more complicated systems.

We feel that much of the confusion associated with vesicle stability, aside from the purity problem mentioned above, stems from the use of light scattering or turbidity measurements as a primary tool. Light scattering is severely limited by the inability to distinguish between aggregation and size transformation. This has often been pointed out (Martin & MacDonald, 1976; Chong & Colbow, 1976; Avramovic & Colbow, 1978; Petersen & Chan, 1978), but the relative contributions have not been evaluated in an unambiguous manner. An equally important problem is caused by the presence of small amounts of multilamellar "contaminants" if the vesicles are not size fractionated before use (Huang, 1969; Barenholz et al., 1977). Marsh et al. (1977) have shown, by light scattering measurements on unfractionated preparations, that the transition due to the small dipalmitoylphosphatidylcholine vesicles, centered around 37 °C (Suurkuusk et al., 1976), cannot be easily distinguished from that due to the larger species at 41 °C.

The great advantage of using light scattering to follow vesicle transformation is, of course, that the sample can be monitored continuously without perturbation. Other methods (electron microscopy, trapped volume, ultracentrifugation, and gel chromatography) rely on the analysis of aliquots, which introduces the possibility of changes in the sample during analysis, and also has a tendency to limit time resolution. ^{1}H NMR has been used to monitor the fatty acid induced transformation of sonicated dimyristoylphosphatidylcholine vesicles (Prestegard & Fellmeth, 1974; Kantor & Prestegard, 1975; 1978) by taking aliquots and measuring the spectra above the transition temperature. More recently, ^{1}H NMR has been used isothermally to monitor Ca^{2+}-induced fusion of phosphatidylcholine–phosphatidic acid vesicles above the transition temperature (Liao & Prestegard, 1979). ^{31}P NMR has also been used to characterize the end product of transformation in both the latter system (Liao & Prestegard, 1979; Koter et al., 1978) and dipalmitoylphosphatidylcholine vesicles (Schullery et al., 1980). In the latter study, the ratio of the number of molecules on the outside of transformed vesicles to that on the inside, measured above the phase transition temperature using the shift reagent Pr^{3+} (Bergelson, 1978),

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